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Increased Lipid Metabolism in Response to Repetitive Aerobic Exercise During Proestrus in Type 1 Diabetes Mellitus Rats

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Abstract

In response to repetitive bouts of aerobic exercise, type I diabetes mellitus (T1DM) female rats demonstrate greater post-exercise blood glucose (BG) recovery during proestrus than T1DM males. Fuel selection differences may explain sex-related differences in the blunted counterregulatory responses to subsequent exercise and estrogen (E2) has shown to mediate fuel metabolism. Evidence of E2's activation of AMP-kinase (AMPK) may mediate the metabolic actions of E2. This study aimed to examine sex-related differences in lipid and glucose metabolism in response to a 4-day aerobic exercise protocol in skeletal muscle and liver tissue of male and female T1DM-induced (n = 5) and non-T1DM (n = 5) rats. Western blot analysis and phenol sulphuric acid and β-oxidation activity assays were used to assess AMPK and gluconeogenic enzyme protein expression, muscle glycogen content, and lipid oxidation. T1DM and non-T1DM females demonstrated significantly greater β-oxidation activity in muscle compared to males but this fuel shift towards lipids was not associated with higher AMPK activity. No muscle glycogen content differences between the sexes were found. T1DM and non-T1DM females demonstrated higher hepatic G6Pase expression than males. Therefore, T1DM females may be less susceptible to repeated exercise-induced hypoglycemia through E2-mediated shift towards lipid oxidation during the proestrus phase.

Keywords

Type I diabetes, exercise, estrogen, fuel selection, lipid metabolism, sex-related differences, AMPK

Summary for Lay Audience

Type I diabetes mellitus (T1DM) is a chronic illness that results in disrupted blood sugar concentrations due to the lack of insulin a hormone which typically helps maintain blood sugar concentrations. A combination of insulin therapy, diet, and exercise can assist the management of T1DM. However, participating in consecutive exercise or exercise training may present a challenge for individuals with T1DM because it can lead to low blood sugar concentrations, or hypoglycemia onset. The typical physiological responses the body produces to maintain blood sugar concentrations are less effective in response to successive aerobic exercise. Interestingly, T1DM females are less affected by these blunted responses to successive bouts of exercise and normalize blood glucose during and following exercise more efficiently than T1DM males. During aerobic exercise of moderate intensity, the body uses a combination of sugars or fats for fuel; however, it has been suggested females use more fats which in turn conserves other energy sources such as sugars for more efficient post-exercise recovery. The sex hormone estrogen has been noted to explain this sex-related difference in fuel selection during exercise. Our laboratory recently demonstrated in response to repetitive bouts of aerobic exercise during elevated concentrations of estrogen blood glucose concentrations in T1DM females post-exercise normalised more quickly than T1DM males. Estrogen has shown to activate a major energy sensor AMP-kinase (AMPK), a known initiator of fat metabolism, and through this enzyme may create a shift towards using fats as fuel during exercise. Additionally, estrogen has shown to decrease gluconeogenesis through expression of key enzymes PEPCK and G6Pase. If this pathway is decreased it may indicate a shift toward fat utilization, however it is unclear how this may influence T1DM as gluconeogenesis is the primary supply of glucose (sugars) during exercise. This thesis investigates sex-related differences in activation of key metabolic regulatory enzymes

involved in fat and glucose metabolism in liver and muscle tissue of male and female T1DM rats following repetitive exercise. We aimed to investigate if differences in fuel selection between sexes and diseased states were attributed to the presence of estrogen. Skeletal muscle and liver tissues were taken on the fourth day of a repetitive exercise protocol which coincided with the phase of high estrogen concentrations (proestrus). The primary findings of this study were that T1DM female rats indeed demonstrated higher β-oxidation activity than T1DM males suggesting increased use of fats as fuel. As tissues were harvested on day 4 during the proestrus phase it can be suggested this shift towards fats may be mediated by the higher concentrations of estrogen. We did not find that the heightened activation of fat metabolism led to the conservation of glycogen stores in muscle and liver tissues. In addition, higher β-oxidation activity was not associated with greater AMPK activation in T1DM females. A greater expression of gluconeogenic enzyme G6Pase in T1DM and non-T1DM females suggests that exercise during higher estrogen did not negatively impact T1DM fuel metabolism, rather an increase in fat availability likely alleviated the challenge to balance blood glucose concentrations. In conclusion, in response to successive aerobic exercise during proestrus T1DM females may be at less risk for hypoglycemia development due to a shift towards fats as fuel, however this was not due to an increase in AMPK, nor did it spare muscle glycogen stores.

Co-Authorship Statement

Dr. Jamie Melling of Western University, London, Ontario, Canada was involved in the development of project organization, interpretation of results, and thesis revisions. Jordan LaRocque was involved in the project design, animal care, and harvesting of tissues.

Dedication

To my family and friends. Thank you from the bottom of my heart for all the love and support.

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To my supervisor Dr. Jamie Melling, from second-year anatomy class to your mentorship throughout this process, thank you for your expert advice, patience, and unwavering support. Your dedication to science and education is and always will be a great inspiration in my academic and personal life. It has been a privilege to have you as my advisor for the past two years. I would also like to thank Dr. Michelle Mottola for her generosity in sharing her expertise and knowledge.

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To my friends: you are my rock, thank you for the unconditional support. Without your encouragement, love, and humour I would not be anywhere near where I am today. To my family: no words will ever suffice. Thank you for always supporting me in every endeavor.

Table of Contents

Abstract ii

Summary for Lay Audience iii

Co-Authorship Statement v

Dedication vi

Acknowledgments vii

Table of Contents viii

List of Figures x

List of Appendices xi

Chapter 1 1

1.1 Overview of Type 1 Diabetes Mellitus 1

1.2 Benefits, Risks, and Counterregulatory Responses to Exercise in T1DM 6

1.3 The Menstrual and Estrous Cycle and the Metabolic Impact of Estrogen 11

1.4 Exercise-Associated Fuel Metabolism in Acute and Repetitive Exercise in Non-Diabetics 15

1.5 Exercise-Associated Fuel Metabolism in Acute and Repetitive Exercise in T1DM 19

1.6 Enhanced Lipid Oxidation Through Estrogen-Mediated Activation of AMPK 22

1.7 Rationale 24

1.8 Purpose and Hypotheses 26

Bibliography 27

Chapter 2 51

2.1 Introduction 51

2.2 Methods and Materials 55

2.2.1. Experimental Design 55

2.2.2. Western Blotting 55

2.2.3. Muscle Glycogen Content 57

2.2.4. Muscle Lysate Assessment of β-oxidation (SCHAD Assay) 58

2.2.5. Data analysis 58

2.3 Results 59

2.3.1. β-oxidation activity 59

2.3.2. AMPK expression and activity 59

2.3.3. Muscle glycogen content 60

2.3.4. Gluconeogenic enzymes 60

2.3.5 Blood glucose concentrations 61

2.3.6. Estrogen concentrations 61

2.4 Discussion 65

2.5 Conclusion 70

Bibliography 71

Appendices 77

Curriculum Vitae 88

List of Figures

Figure 1. *β-oxidation, SCHAD activity assessment of skeletal muscle in male and female T1DM and control rats. Mean muscle SCHAD activity (μmol·g wet wt^{-1}·min^{-1}) from day 4. All data are presented as mean ± SEM. There was a main effect of sex (P = 0.0326).* 61

Figure 2. *Western blot quantification and representation blots of AMPKα and p- AMPKα2 from day 4 of exercise protocol for control and diabetic exercise males and females. (a) AMPKα (b) p- AMPKα2 (c) Phosphorylated ratio and (d) Representative blots relative to β-actin control. All data are presented as mean ± SEM. * denotes significance. There was a main effect of sex (P = 0.0242) and diabetes (P = 0.0244) for AMPKα. There was a difference between DXF and CXM in AMPKa (P = 0.0145). There was no difference in AMPKa between CXF and CXM (P > 0.05) or between DXF and DXM (P > 0.05). There were no significant differences in p- AMPKα2.* 63

Figure 3. *Muscle glycogen content measured in (umol/g) from day 4 of exercise for control and diabetic exercise males and females. All data are presented as mean ± SEM. There was no significant difference between CXF and CXM or between DXF and DXM (P > 0.05).* 63

Figure 4. *Western blot quantification and representative blots for gluconeogenic enzymes G6Pase and PEPCK. (a) G6Pase protein content quantification (b) PEPCK protein content quantification and (c) Representative blots relative to β -actin control. All data are presented as mean ± SEM. * denotes significance (P < 0.05). There was a main sex effect in G6Pase (P = 0.0080). DXM (P = 0.0173) and CXM (P = 0.0262) were significantly lower than CXF. There were no significant differences in PEPCK.* 65

List of Appendices

Appendix A: Mean blood glucose response from day 4 of exercise protocol

Appendix B: Serum estrogen concentrations from day 1 and day 4 of exercise protocol

Appendix C: Liver glycogen content from day 4 of exercise protocol

Appendix D: Western Blotting Protocol

Appendix E: Phenol-Sulphuric Acid Assay

Appendix F: β-oxidation, short-chain β-hydroxyacyl-CoA dehydrogenase (SCHAD) Assay

Appendix G: Bradford Protein Assay

Chapter 1

1.1 Overview of Type 1 Diabetes Mellitus

Diabetes is an umbrella term used to describe two prevalent metabolic disorders relating to blood glucose (BG) homeostasis. Both Type I diabetes mellitus (T1DM) and Type II diabetes mellitus (T2DM) lead to impaired glycemic control and are characterized by chronic high BG concentrations (*CONCENTRATION*) (hyperglycemia) resultant of deficient insulin secretion, action, or a combination thereof[1]. The more prevalent of the two diseases, T2DM accounts for approximately 90% of diagnoses in Canada and results from a combination of genetic, lifestyle, and environmental factors[2]. Conversely, T1DM constitutes the remaining 10% of diabetes diagnoses in Canada and is the result of an autoimmune reaction[3]. The etiology of T1DM is complex and although the cause is commonly linked to family history, evidence demonstrates a less than 50% genetic concordance of T1DM in monozygotic twins suggesting other influences play a role such as environmental factors[4]. Although currently most diabetes cases are predominantly T2DM, the prevalence of T1DM is expected to increase by 5-10% between 2020 and 2030 in Canada as result of environmental factors which influence the pathogenesis such as vitamin D deficiency and viral infections (e.g., enterovirus) [3, 4]. Similarly, projections in the US suggest that the incidence of T1DM in individuals younger than 20 years of age will increase 23% by 2050[4]. With these significant surges in diagnosed T1DM, it is predicted it will directly cost the Canadian health care system 4.9 billion dollars to manage diabetes by 2030, presenting a significant public health and economic burden[3]. Additionally, in conjunction with the COVID-19 pandemic, public health stress is likely to rise as new studies strongly suggest pre-existing diabetes is

proposed to be one of the highest risk factors for developing COVID-19[5]. Evidence also suggests COVID-19 can potentially induce new on-set T1DM and promote the generation of autoantibodies against pancreatic β -cells. COVID-19 can also trigger severe diabetic ketoacidosis, which has a 4 to 10% mortality rate[5, 6]. With predictions that COVID-19 will become an endemic and the increasing prevalence of diabetes, now more than ever research efforts need to focus on preventative and interventive tools for the management of T1DM[6, 7].

The pathophysiology of diabetes is rather complex and distinct between the two major classifications of the disease. In 1984, George Eisenbarth developed a theoretical model for the pathogenesis of T1DM[8, 9]. It highlights the progression of the disease in a single line trajectory starting with predisposed genetic risk, followed by an environmental trigger that disrupts islet-specific functioning which ultimately leads to pancreatic B-cell loss and disrupted BG homeostasis[9, 10]. Although Eisenbarth's model is still used today, the complexities of the pathogenesis of T1DM continue to be elucidated which has led to considerable alterations to the model in order to consider the heterogeneities per stage of T1DM progression[10].

In diabetes mellitus, the innate physiological mechanisms which maintain a narrow range of BG concentrations are disrupted[2, 4]. Specifically, due to dysfunctional pancreatic β-cell action and insulin resistance, individuals with T2DM have impaired insulin secretion and action[1, 11]. As a result, despite circulating insulin, glucose homeostasis is dysregulated, and the body is unable to efficiently use glucose from the bloodstream for energy processes resulting in hyperglycemia[12]. Conversely, in T1DM T-cell mediated destruction of pancreatic β-cell leads to complete β-cell loss and ultimately insulin

deficiency and hyperglycemia[1, 13, 14]. Accompanying pancreatic β-cell destruction, pancreatic α-cells are also dysregulated in T1DM resulting in elevated glucagon concentrations[14, 15] . In non-diabetic physiological conditions, in response to low BG concentrations (hypoglycemia) pancreatic α-cells secrete the hormone glucagon to raise BG concentrations[13]. Typically, in response to hyperglycemia glucagon is suppressed, however in T1DM glucagon continues to increase BG concentrations further exacerbating metabolic disruption due to insufficient insulin[1, 13]. Diabetic ketoacidosis is an example of metabolic disruption that can present as a result of continued glucagon secretion paired with insulin deficiency[16]. If left untreated, T1DM leads to uncontrolled lipolysis and elevated concentrations of free fatty acids (FFAs) in the blood stream due to insufficient insulin to synthesize and store triglycerides in adipose tissue[1]. FFAs are metabolized into ketone bodies in the liver and an increased availability of FFAs and ketone bodies disrupts glucose metabolism by reducing the utilization of glucose. Excessive production of ketone bodies can increase the acidity of the individuals blood which may be fatal, especially in children[17]. Nevertheless, since the discovery of insulin, T1DM has transformed from a fatal to treatable disease[18]. Survival and overall health of the patient with T1DM has significantly improved over the decades, however the management of T1DM remains unoptimized and is associated with life-threatening complications[19, 20]. In part, this is due to the complexity of its etiology and the difficulty in timely diagnosis[10].

Historically, T1DM was considered a disorder in children and adolescents, however more recently this opinion has changed as it is evident that nearly 50% of T1DM diagnoses are in adulthood[10]. This is also supported by the fact that 50% of T1DM

diagnoses are initially misdiagnosed as T2DM[4]. Now, clinicians deter from age at onset as a primary symptom to diagnose T1DM and various methods are used for proper diagnosis. Typically, the general diagnosis of diabetes includes a "fasting BG higher than 7 mmol/L, a random BG concentration of 11.1 mmol/L or higher with symptoms of hyperglycemia, or an abnormal 2-hour oral glucose-tolerance test (OGTT)"[10, 21]. In 2009, a glycated haemoglobin (HbA_{1c}) concentration over 6.5% was introduced in the diagnosis guidelines[8, 10]. Haemoglobin is contained in red blood cells which survive up to three months, therefore this measurement represents chronic glycemic control over several months[22, 23]. The use of HbA_{1c} as a biomarker for T1DM is promising as a 20% increase in HbA1c has shown to be associated with an 84% increased risk for T1DM within three years[4]. More recently, a new staging model was introduced to account for the preclinical stages of T1DM including the measurement of autoantibodies specifically against β-cell proteins[4, 24]. Stage one includes the classification of β-cell autoimmunity which is defined as the detection of more than one islet autoantibody (AAbs), leading to stage two where glucose intolerance is presented, and finally stage three is the clinical manifestations of the disease[25]. There appears a clear relationship between increasing detectable AAbs and the incidence of symptomatic diabetes as the Environmental Determinants of Diabetes in the Young study demonstrated that 5-year incidence of symptomatic T1DM was 47% higher in children with three AAbs[26]. Additionally, low C-peptide concentration, an indicator of severe endogenous insulin deficiency, is used as an initial and on-going assessment of diabetes[10, 27]. The Diabetes Control and Complications Trial (DCCT) found an inverse relationship between C-peptide concentration and short- and long-term complications related to diabetes[28]. Currently, the best predictor of T1DM within a year

before diagnosis is a combination of islet AAbs count and abnormal OGTT[29]. Thus, the timely diagnosis of T1DM is significant such that the condition can immediately be managed through insulin therapy and lifestyle intervention[10].

Following the advancement of insulin therapy, in the 1990's the DCCT was conducted to examine the benefits of intensive insulin therapy (IIT) versus conventional therapy (CT)[28]. A more traditional and generally interventive approach, CT consists of a one to two daily insulin injections per day to prevent hyperglycemia while not inducing hypoglycemia[28, 30, 31]. Conversely, IIT is more extensive and requires multiple, smaller doses throughout the day which are adjusted based on continual measures of BG concentrations through self-administered BG tests[30]. A more preventative approach mirroring physiological insulin secretion and action, IIT attempts to achieve a more consistent and normalized BG concentration throughout the day and a target HbA_{1c} of less than 7%[30]. Overall, the findings of the DCCT concluded that individuals using IIT had tighter glycemic control and lower HbA_{1c}[28]. IIT users had a substantial reduction in the occurrence of micro- and macrovascular complications such as decreased risk and progression of retinopathy, neuropathy, and nephropathy[28, 30]. However, although IIT was more beneficial for alleviating some disease-associated risks, there was a threefold increase in the risk of hypoglycemia when compared to CT[28, 32–34.] Clinically, hypoglycemia is defined as a BG below 3.0 mmol/L, however symptoms of hypoglycemia such as loss of consciousness and seizures manifest at concentrations of 3.9 mmol/L[35, 36]. Hypoglycemic events pose life-threatening complications with adverse effects on cognitive function and are associated with 4-10% of T1DM-related deaths[10, 37]. The strongest predictor of hypoglycemia is a precedent bout of severe hypoglycemia,

therefore the use of IIT, although considerably beneficial for day-to-day living, should not be exclusively used to treat and manage T1DM[34, 36].

1.2 Benefits, Risks, and Counterregulatory Responses to Exercise in T1DM

Exercise for the management of T1DM is associated with many positive benefits including enhanced insulin sensitivity, lower blood pressure, reduced insulin dosage requirements, and increased overall well-being[36, 38, 39]. Regular participation in physical exercise is recommended to minimize the complications associated with T1DM; improving cardiovascular disease risk marker, blood lipid profiles, and overall bone health, as well as lowering the risk of diabetic neuropathy and nephropathy[36, 39]. In addition, there is evidence in children with T1DM exercise training can reduce HbA_{1c} concentrations, however this effect is not conclusive in adults[40, 41]. Yet, 63% of adults with T1DM do not meet current physical activity guidelines nor do they achieve a healthy body weight[42]. Inactivity among this patient population leads to lower skeletal muscle health and insulin resistance development, impaired glucose and lipid disposal, and reduced basal metabolic rate[39, 42, 43]. Loss of bone mineral density and osteoporosis are also common in inactive T1DM individuals, even in the presence of well-controlled BG concentrations[42]. Evidently, exercise for the management of T1DM is beneficial and should be incorporated into individuals' daily lifestyle. However, unlike non-diabetic individuals who can seemingly choose any mode of exercise to practice, recommending the safest frequency, intensity, duration, and type of exercise for T1DM is challenging due to altered neuroendocrine and hormonal (counterregulatory) responses to exercise[36, 39, 42].

Current exercise guidelines for individuals with T1DM recommend 150 minutes of moderate-intensity or 90 minutes of vigorous-intensity exercise per week with no more than two consecutive sedentary days[40, 44]. Research efforts on the benefits of exercise for T1DM have predominantly focused on acute and chronic aerobic training[38]. Evidence strongly suggests aerobic exercise reduces many disease-associated complications and significantly improves glycemic control[45–47]. Aerobic exercise has also shown to decrease insulin dosage requirements, improve lipid concentrations, and cardiovascular fitness[48–50]. A meta-analysis on exercise and T1DM concluded that aerobic exercise training for longer than three months can decrease HbA_{1c} concentrations particularly in individuals with an initial HbA_{1c} concentration greater than 8%[51]. Aerobic exercise has also shown to improve skeletal muscle components such as muscle capillarization and oxidative capacity, potentially providing significant metabolic benefits[42, 52]. A 10-week moderate-intensity aerobic exercise intervention demonstrated enhanced insulin sensitivity while another study demonstrated a decrease in insulin dosage after aerobic exercise training[50, 53]. Despite increasing research efforts for the development of exercise strategies for T1DM, as aforementioned, most individuals with T1DM remain inactive and this is largely due to the fear of exercise-induced hypoglycemia[35]. Understandably, after a 45-minute exercise session the risk of hypoglycemia during and post-exercise increases by 30-45%, lasting up to 31 hours as a result of exercise-induced increase in insulin sensitivity and increased glucose uptake (i.e., muscle glycogen repletion) [35, 42].

Exercise-induced hypoglycemia is a severe risk for individuals with T1DM, however it is not a phenomenon exclusive to T1DM. Depending on the intensity, duration, and pre-exercise meal, under normal physiological conditions hypoglycemia may occur but

rarely ever presents severe risk due to the many counterregulatory mechanisms which prevent and correct hypoglycemia[54, 55]. Typically, during moderate-intensity exercise, individuals without T1DM exhibit a reduction in insulin secretion that is paired with an increase in glucagon to ensure the rate of glucose utilization by the exercising muscle is equally matched to glucose provision[56]. In response to fluctuating BG concentrations, glucagon stimulates liver glucose release via glycogenolysis and gluconeogenesis (GNG) and if a reduction in BG persists additional hormones and catecholamines such as epinephrine, norepinephrine, cortisol, and growth hormone are released in a sequential pattern[57, 58]. These hormones also take part in stimulating hepatic glucose production and induce fatty acid release from adipose tissue. Overall, to ensure sufficient energy is provided to exercising muscles, these counterregulatory responses initiate metabolic signaling pathways which produce energy predominantly from fatty acids and glucose[59, 60]. The result is relatively stable BG concentrations that can be sustained for several hours without the need for food intake[42].

Counterregulatory responses to exercise in individuals with T1DM are reduced leading to a mismatch between glucose utilization to glucose provision. In T1DM, as a result of β-cell loss and function, exogenous insulin is required to maintain BG homeostasis[42]. Unlike endogenous insulin which has a half-life of approximately 5 minutes, exogenous insulin has a significantly higher half-life lasting up to hours[42, 61]. Therefore, the concentrations of insulin prior exercise are dependent on the timing and quantity of insulin therapy taken by the patient. Due to the timing and peak action of exogenous insulin, it is likely individuals with T1DM have higher concentrations of insulin prior exercise[42, 61]. Elevated insulin may suppress the glucagon response to

exercise and limit glucose production by the liver namely GNG while stimulating glucose uptake and storage into respective peripheral non-exercised tissues[62]. As a result, the drop in BG concentrations are potentiated further in the post-exercise period as muscle glycogen stores are replenished leading to an increased risk of hypoglycemia development[42, 61, 62]. Current recommendations for exercise and T1DM include increasing carbohydrate consumption prior to exercise and the alteration of insulin dosage and timing, as two key preventative strategies to reduce the risk of exercise-induced hypoglycemia[36]. However, there is a tendency for individuals with T1DM to overcompensate for exercise by consuming excessive amounts of carbohydrates and/or decreasing insulin doses. This may negate the beneficial effects of exercise by inducing weight gain from overconsumption and potentially worsen BG control by altering insulin therapy[36]. In summary, counterregulatory responses in T1DM are altered due to a combination of the concentration, timing and actions of exogenous insulin, dysregulated glucagon response, inadequate hepatic glucose production, excessive pre-exercise carbohydrate consumption, and post-exercise glycogen recovery[36, 42, 62, 63].

Not only are counterregulatory responses to exercise disrupted in T1DM, but there also appears to be a sex-related difference in individuals with and without T1DM[64–68]. At moderate intensities, the neuroendocrine response to exercise appears to be greater in men[65]. During euglycemia and hypoglycemia, men with and without T1DM demonstrate an elevated concentration of epinephrine and glucagon during and following exercise in comparison to exercised women[65, 66]. Women with and without T1DM have lower release concentrations of counterregulatory hormones including glucagon, epinephrine, norepinephrine, and growth hormone, with a concomitant reduction in hepatic glucose

production following exercise[65, 69]. Interestingly, despite reduced release concentrations of these counterregulatory hormones, it has been suggested women have greater sensitivity to the action of these hormones and thus require lower concentrations to elicit an equal response[65, 70]. Moreover, counterregulatory responses appear to be reduced with subsequent exercise in individuals with and without T1DM and of both sexes[67, 68, 71].

To implement exercise regimen strategies repetitive bouts of exercise (i.e., daily) must be considered. Through exercise or insulin therapy correction, antecedent periods of hypoglycemic stress results in blunted counterregulatory responses to subsequent glycemic challenges in both sexes[70]. However, counterregulatory responses in men are suppressed twofold when compared to women in response to repeated hypoglycemia[70]. Several mechanisms have been proposed to explain these sex-related differences in counterregulatory responses during repeated bouts of hypoglycemia, and some research efforts have highlighted differences in substrate metabolism and the important role of female reproductive hormones in this response[66, 70, 72]. Specifically, the role of estrogen (E2) in metabolism has been well documented in studies conducted in animal models, women with menopause, and performance studies in men supplemented with E2[73–75]. It has been suggested that women may be less susceptible to the blunted counterregulatory responses to repeated hypoglycemia through fuel selection differences, particularly with an increased reliance on lipid metabolism[66, 70]. Interestingly, the mediated actions of E2 on metabolism have demonstrated enhanced lipolysis and lipid oxidation while reducing lipogenesis[73, 76, 77].

1.3 The Menstrual and Estrous Cycle and the Metabolic Impact of Estrogen

With respect to the ovarian cycle, the menstrual cycle can be simplified into two phases: the follicular phase (FP) and the luteal phase (LP)[76, 77]. Although there are individual variations due to genetics, metabolic conditions, age, and race, reproductive hormones fluctuate in a predictable manner throughout the menstrual cycle[78–81]. Estrogen (E2), progesterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) all characteristically rise and fall per menstrual cycle phase[76]. A typical, or 'textbook', menstrual cycle starting from the first day of menses to the beginning of the next menses in young, healthy women averages 28 days. The FP, which is more prone to length variation than the LP, is approximately 12-14 days, where ovulation occurs on the 14th day[76, 82]. The LP averages 14 days and is characterized by an increase in E2 concentrations. This increase in E2 suppresses the release of pituitary hormones LH and FSH, while progesterone is released from the corpus luteum and increases throughout the LP[82]. Menses initiates when there is no fertilization and E2 concentrations start to decrease. To initiate the maturation of new follicles, FSH is released and as the follicle matures E2 concentrations slowly rise eliciting positive feedback to increase LH concentrations marking the beginning of the LP[76, 77, 82].

E2 is increasingly recognized to be an important regulator of energy homeostasis and metabolism, particularly in glucose and lipid metabolism[73]. The metabolic actions of E2 in skeletal muscle, liver, and adipose tissue improves insulin sensitivity and glucose tolerance and alters substrate metabolism at rest and during exercise in men and women[73, 83–85]. Among eumenorrheic women, E2 functions as a circulating steroid hormone that

acts on target tissues and mediates its effects by binding to three main estrogen receptors (ER) – E2 receptor α (ERα), E2 receptor β (ERβ), and the most recent discovery, G protein-coupled E2 receptor 1 (GPER)[85]. ERs transmit physiological signaling in response to E2 and have distinct expression patterns in tissues including the liver, adipose tissue, skeletal muscle, and breast tissue[86]. Primarily produced in the ovaries, E2, in its most common form 17β-estradiol, is carried through the bloodstream to target tissues where it binds and activates ERs acting as a transcription factor to regulate gene expression of important cellular factors involved in growth, differentiation, and homeostasis[83, 87, 88]. ERs act by regulating transcriptional processes through various signaling mechanisms[85]. There are two mechanisms in which E2 exerts its actions, the genomic or nongenomic signaling pathway[89]. The genomic effects of E2 are mediated by the nuclear translocation of an E2-ER complex which directly binds to estrogen response elements on the promoter region of targeted genes[88, 90]. Alternatively, the E2-ER complex can bind to transcriptional coactivators to induce gene transcription indirectly[91]. Nongenomic E2 signaling results in more rapid effects of the hormone whereby the E2-ER complex, or E2 bound to GPER (non-nuclear, membrane-associated ER) activate signaling molecules such as cyclic-AMP or phosphoinositide 3-kinase[92–94]. Protein kinase cascades alter the function of proteins in the cytoplasm leading to no gene regulation but instead indirectly influences gene expression through transcription factors[87].

Due to the ability to assess novel therapies and investigate a large range of molecular mechanisms, research on the effects of E2 has predominantly been conducted in animal models particularly in rodents[95–97]. The estrous cycle in rodents is equivalent to the menstrual cycle in humans and is comprised of four phases (proestrus, estrus, metestrus,

and diestrus), repeating every 4 to 5 days. Metestrus and diestrus typically have the highest concentrations of progesterone while proestrus is recognized for higher concentrations of E2 which remain elevated for 12 hours[95, 96].

Rodent models are especially helpful to delineate the pathogenesis of metabolic disorders and many diabetes mellitus rodent models present sexual dimorphism in glucose regulation and associated complications[97, 98]. For example, as similarly observed in human patients, chronic hyperglycemia is pronounced in males while females remain more normoglycemic[98]. To thoroughly investigate the role of reproductive hormones and particularly the metabolic impact of E2, ovariectomized (OVX) rodents are commonly used. OVX models have consistently shown altered glucose homeostasis by impaired glycemia and insulin secretion and reduced glucose tolerance[99, 100]. Additionally, other models such as ERα or aromatase knockout mice, resulting in ablated receptor signaling and depleted estrogen synthesis, are characterized by reduced lipid oxidation, hyperinsulinemia, and increased adiposity and hepatic glucose output[101]. Menopausal and postmenopausal women, characterized by the gradual systemic loss of E2, demonstrate similar metabolic phenotypes including increased adiposity, decreased insulin sensitivity, and the development of insulin resistance[102, 103]. Moreover, E2 supplementation studies in rodent and human models with E2 deficiency, such as OVX rats and women with menopause, have demonstrated favourable outcomes in whole-body metabolic profile including improved systemic insulin sensitivity, reduced adiposity, and improved glucose tolerance[74, 104, 105].

A clear sexual dimorphism is present in neuroendocrine and metabolic responses to exercise. As aforementioned, women are less susceptible to the blunted counterregulatory

responses to repeated hypoglycemia, and this may be explained by differences in hormone concentrations and fuel selection. At rest, there are no apparent differences in fuel selection between men and women, however throughout various types of exercise it is well established women with and without T1DM rely more on lipid metabolism, demonstrated by lower respiratory exchange rate (RER), whereas men favour carbohydrate oxidation for fueling exercising muscles[66, 70, 106]. Additionally, female skeletal muscle has consistently shown more capacity for lipid transport, intramyocellular triacylglycerol (IMTG) production, and β-oxidation[103]. This is supported through higher expressions of genes associated with fatty acid transport and synthesis (i.e., FAT/CD36, lipoprotein lipase, carnitine palmitoyl transferase I (CPT I))[107]. This predominant use of lipids as fuel source is also related to E2-induced inhibition of gluconeogenic-mediated hepatic glucose output and lower depletion of hepatic and muscle glycogen[105]. Moreover, E2 supplementation in exercising men has resulted in a shift towards lipid utilization through significantly lower RER [66, 74]. OVX rats demonstrate an increase in muscle and hepatic glycogen use during a 4-week exercise regimen and E2 supplementation reversed this effect by attenuating glycogen use and increasing lipid oxidation[75]. Another study in OVX rats demonstrated similar results whereby there was an increased availability of lipids for oxidation and spared glycogen with E2 administration over a 2-week period [106].

Fluctuating hormones concentrations during the menstrual cycle are believed to influence the metabolic responses to exercise depending on the phase of the menstrual cycle they are tested in[108]. Typically, women are tested during the FP because hormone concentrations, particularly E2, are relatively stable and similarly observed to concentrations in men[66]. However, these methodologies limit the influence of E2 on

study outcomes. Studies examining exercising women during the LP have shown there is a higher concentration of circulating E2 elucidating a higher relative rate of fatty acid oxidation (FAO) in comparison to the FP[107–109]. Furthermore, phase specific differences are evidenced through increased preservation of total and muscle glycogen in women exercising in LP, while women exercising in FP and men have similar glycogen use[107]. The literature supports the increasing availability of lipids for substrate oxidation and glycogen sparing during exercise, particularly in LP, which can be attributed to the influence of E2.

1.4 Exercise-Associated Fuel Metabolism in Acute and Repetitive Exercise in Non-Diabetics

Exercise-associated fuel metabolism involves an integrated system between various tissues, however the main tissues for energy provision and utilization are the liver, skeletal muscle, and adipose tissue[110–113]. Increasing energy demands from exercise necessitate an increase in energy marked by increased glucose uptake in the muscle and increased utilization of lipids and glycogen[110, 114]. The proportion of fuel source provided by the liver, adipose tissue, and the muscle itself is a delicate balance and depends on many factors. For the purpose of this overview, the contribution and processes of respective tissues will be explained in the context of moderate-intensity exercise as evidence supports a modest amount of moderate-intensity is adequate for significant health benefits[115].

Once exercise commences glucose utilization immediately rises in the muscle with the breakdown of glycogen stores (glycogenolysis)[113, 116]. Rapid uptake of glucose from the blood also increases via a 20-fold increase in blood flow to the exercising muscle[113,

[117]. During exercise, increased glucose production from the liver continues to play a major role in maintaining plasma BG concentrations[114, 118]. The main substrates and the proportion of each substrate utilized during exercise largely depends on the intensity and duration of the exercise performed[110, 113, 119]. Carbohydrates in the form of muscle glycogen and plasma BG, and lipids from plasma FFAs and IMTGs are the primary fuel sources for exercising muscles[116]. Lower intensity exercise (~25% $VO2_{max}$) relies more on lipids for energy and as exercise intensity increases (~65% $VO2_{max}$) plasma FFAs decrease while IMTG oxidation increases[110, 113, 119, 120]. However, lipids cannot be sufficiently oxidized to match the rate of energy utilization, therefore the other half of energy is derived from carbohydrate oxidation mainly from muscle glycogen[112]. Heavy-intensity exercise (~85% $VO2_{max}$) is primarily fueled by muscle glycogen and although the rate of lipid oxidation decreases, IMTGs directly release FFAs into the cytosol of the muscle avoiding the muscle plasma membrane for quick use[110, 112]. Fuel utilization becomes more complex with the inclusion of a pre-exercise meal. A rich carbohydrate meal prior exercise, a common practice among individuals with T1DM to attenuate the risk of hypoglycemia, blunts FFA mobilization, increases muscle glycogen, and typically enhances exercise performance[121].

Skeletal muscle metabolism is in part dictated by individual fibre type that comprise each muscle and this composition can change with diet, exercise, and disease conditions[122, 123]. Muscle fibre type can be divided into two categories, type 1 and type 2 fibres and this distinction is based on the different myosin heavy chain isoforms. However, muscle fibres were initially distinguished as white and red muscle based on the different amounts of myoglobin present[122]. Now characterized as slow twitch or type 1

and fast-twitch type 2a, red muscle fibres are mitochondrial-rich and are more resistant to fatigue and better suited to continuous aerobic movement. Alternatively, white muscle fibres or fast glycolytic 2x fibres (predominant in humans) and 2b fibres (predominant in rodents) have less mitochondria thus produce ATP via glycolysis and are more suited to fast movements. During continuous, moderate-intensity exercise type 1 fibres are predominantly recruited and have a great capacity to oxidize lipids for fuel[122, 123].

At the onset of exercise, contraction-mediated cellular events facilitate the translocation of GLUT4 to the sarcolemma in skeletal muscle for glucose uptake from circulation[124]. Skeletal muscle lipid metabolism increases with the use of lipids in the form of FFAs and triglycerides (TG). To facilitate the availability of lipid sources, an increase in blood flow and transport into the muscle are required[113]. Additionally, a reduction in insulin concentrations is required to release its inhibitory effect on the activity of proteins responsible for the expression of genes involved in lipid availability[116, 122]. Derived from adipose tissue, FFAs are transported through fatty acid translocase (FAT/CD36) into muscle, allowing the uptake of long-chain fatty acids (LCFA). Alternatively, derived from circulation or wrapped in very-low-density-lipoproteins from the liver, TGs are hydrolyzed by lipoprotein lipase and are increasingly used as exercise intensity increases[119, 124]. FAT/CD36 is more predominant in type 1 fibres and its expression at the membrane is increased in response to exercise training[125, 126]. Once transported into the muscle, FFAs are either directed to the mitochondria for oxidation or stored as IMTGs[112]. The rate-limiting enzyme CPT1 is essential for mitochondrial oxidation and converts LCFA-CoA into acyl carnitine to cross the inner membrane[127]. Malonyl CoA is a known inhibitor of CPT1 and is produced by the enzyme

acetyl-CoA carboxylase (ACC) and degraded by malonyl CoA decarboxylase (MCD)[127]. 5' adenosine monophosphate-activated protein kinase (AMPK) inactivates ACC and contraction can activate MCD. Therefore, any mechanism which increases AMPK activity releases the inhibition on CPT1 promoting lipid oxidation in muscle[128]. Exercise alters intracellular AMP:ATP ratio and reduces intramuscular glycogen concentrations, both known activators of AMPK, therefore during exercise lipid oxidation should increase by way of increased AMPK activity[129, 130]. It has been well documented many of the changes in lipid-based metabolism during exercise are directly mediated by AMPK[130].

While the muscle uptakes and oxidizes energy between carbohydrates and fats, the liver matches the utilization with increased mobilization of hepatic glycogen and GNG to maintain blood glucose homeostasis[114, 131]. In T1DM, GNG is the predominant pathway for hepatic glucose production during exercise[132]. At moderate-intensity exercise, determined primarily by glucagon action, GNG is a process that transforms non-carbohydrate substrates into glucose including lactate, amino acids, and glycerol[131]. For instance, pyruvate derived from lactate and alanine enters the mitochondria to be carboxylated into oxaloacetate (OAA). OAA is then reduced to malate to enter the cytoplasm where its reoxidized into its original form and then transformed into phosphoenolpyruvate (PEP) by a rate-limiting enzyme PEP carboxykinase (PEPCK). PEP then enters the gluconeogenic cycle to ultimately be converted into glucose-6-phosphate (G6P). Finally, G6P is converted into glucose via another rate-limiting enzyme glucose-6-phosphatase (G6Pase)[118, 131, 132]. Other precursors such as glycerol are converted into glyceraldehyde-3-phosphate and enter the gluconeogenic cycle while

amino acids are converted into specific intermediates in the tricarboxylic acid cycle before entering the gluconeogenic cycle[131, 133]. Although GNG is simplified as the reversal of glycolysis (the breakdown of glucose into energy) there are four unique gluconeogenic enzymes essential for overcoming irreversible glycolysis reactions[132]. In particular, PEPCK and G6Pase are significantly important to GNG and their deficiency contribute to liver dysfunction and glycogen storage disease, along with hypoglycemia and hyperlipidemia[132]. Increased FFA availability and oxidation has been associated with an increase in GNG while the inhibition of lipolysis has shown to dramatically decrease this pathway[134]. Interestingly, males demonstrate higher basal content of both PEPCK and G6Pase while females may present reduced rates of GNG due to the influence of sex steroid hormones such as estrogen[135–137]. Studies have demonstrated that E2 reduces the expression of PEPCK and G6Pase thereby downregulating GNG[137, 138].

1.5 Exercise-Associated Fuel Metabolism in Acute and Repetitive Exercise in T1DM

Similar to individuals without T1DM, the fuel source for exercising muscles in T1DM depends on the duration and intensity of exercise[42]. However, abnormalities in fuel metabolism during exercise in T1DM have been recognized and appear to depend on the state of BG determined by exogenous insulin concentrations[42, 62, 122]. As previously described in section *1.2,* alterations in neuroendocrine and hormonal responses to exercise in individuals with T1DM are primarily attributed to deficient endogenous insulin and/or excessive exogenous insulin. Hepatic glucose production may be inhibited due to the likelihood of elevated concentrations of insulin at the onset of exercise due to exogenous insulin therapy and increased insulin sensitivity[62]. In individuals with T1DM

that are deprived of insulin for 12-24 hours, prolonged moderate-intensity exercise is associated with lower RER and consequently a reduced rate of carbohydrate oxidation in comparison to non-T1DM at the same exercise intensity[139]. It is postulated that individuals with T1DM rely more on lipid oxidation during exercise when insulin concentrations are low than when they have elevated insulin concentrations, mirroring normal physiological conditions[139, 140]. Alternatively, in hyperinsulinaemic conditions substrate metabolism in individuals with T1DM is marked by low lipid oxidation and increased muscle glycogen utilization presenting risk for early fatigue[140]. A comparison of individuals with T1DM exercising in euglycemia versus hyperglycemia concluded the latter condition relies heavily on carbohydrates while the former reveals a shift towards lipid oxidation[140]. This suggests that substrate oxidation in individuals with T1DM performing aerobic exercise in euglycemia is comparable to that observed in non-T1DM, demonstrating a shift towards lipid oxidation throughout the course of exercise. Therefore, when the concentrations of insulin are moderately controlled and circulating concentrations are near physiological concentrations, the ratio of carbohydrate to lipid utilization during exercise in T1DM is similar to that of individuals without diabetes[62]. However, achieving tight glycemic control is challenging for individuals with T1DM as 30 to 50% of diabetes patients do not meet glycemic control targets[141]. Typically, in non-diabetic subjects, insulin concentrations decline considerably to 30 pmol/L or less during exercise, whereas the average pre-prandial insulin concentration in individuals using a basal bolus insulin regimen is typically 120-180 pmol/L[142, 143]. Therefore, depending on the exercise timing and peak action of therapeutic insulin, individuals with T1DM will likely be carrying out exercise in hyperinsulinaemic conditions.

Although acute exercise studies are beneficial to understand specific processes and mechanisms, repeated exercise or exercise training studies are more applicable to prescribing an exercise regimen. Exercise training is well known to elicit adaptations in fuel metabolism[144–146]. A trained state adaptation has consistently shown increased reliance on lipid oxidation during submaximal exercise while sparing carbohydrate sources (e.g., muscle glycogen)[147]. This dependence on lipid oxidation is reflected by lower RER and is interestingly associated with lower plasma FFA concentrations suggesting trained muscles may utilize plasma FFAs more rapidly than untrained muscles[148]. Regular exercise has also shown to increase GLUT 4 tissue concentration in muscle leading to improved glucose disposal and glycogen storage[149]. Although some studies have investigated exercise-associated fuel metabolism in T1DM and strategies for T1DM athletes have been developed, it has not been thoroughly investigated if exercise training elicits similar adaptations in fuel metabolism in T1DM as seen in nondiabetic individuals[150, 151]. However, as aforementioned in section *1.2*, in response to subsequent exercise, the counterregulatory responses to hypoglycemia may be preserved in females with and without T1DM in comparison to males. During exercise, when BG concentrations are reduced below 3.8 mmol/L counterregulatory responses are observed and hormones such as growth hormone and cortisol are secreted to increase lipolysis and FFA mobilization[42, 152].

Neuroendocrine and hormonal responses are significant coordinators of fuel utilization. Evidence suggests that males demonstrate suppressed counterregulatory responses to repeated hypoglycemia in comparison to females with and without T1DM and this may be due to fuel partitioning differences in response to exercise[65]. The

physiological mechanisms underlying these differences are inconclusive, but evidence supports the reproductive hormone E2 has a contributory role particularly in lipolytic metabolism[70, 105]. Interestingly, evidence has shown E2 can activate AMPK in skeletal muscle potentially explaining its lipolytic metabolic actions and supporting the plausibility that women may be protected against acute or repeated exercise-induced hypoglycemia through a stronger reliance on lipid metabolism in comparison to men[153].

1.6 Enhanced Lipid Oxidation Through Estrogen-Mediated Activation of AMPK

E2 is associated with a shift in fuel usage towards increased lipid oxidation while reducing carbohydrate metabolism and sparing glycogen stores[74, 75, 104, 154]. As a result, this increased flexibility in substrate utilization may be a mechanism which females with T1DM favour to preserve the counterregulatory response following prior hypoglycemia. A recent study from our laboratory demonstrated that females with T1DM had greater post-exercise BG recovery in response to repetitive exercise-induced hypoglycemia in comparison to males[155]. This study was staged such that the last day of exercise coincided with the proestrus phase of the estrous cycle (high E2). It can therefore be suggested T1DM females were able to recover and stabilize BG post-exercise quicker than males as a result of E2-mediated actions on fuel selection.

E2-induced activation of AMPK, particularly its α-catalytic subunit, has been noted in various tissues including skeletal muscle, adipocytes, hepatocytes, endothelial cells, cancer cell lines, and myotubes[153, 156–160]. An important mediator of the beneficial metabolic effects of exercise, the mechanisms of AMPK activation have been well investigated with various findings[161–166] A particular study assessed the potential role of

AMPK activation in mediating the metabolic effects of E2, mainly in lipid metabolism[153]. AMPK phosphorylation (Thr-172) was assessed in muscle, liver, and adipose tissue of OVX mice in a control or E2 supplemented condition. A significant increase (~5-fold) in AMPK phosphorylation was evident in skeletal muscle of OVX mice treated with E2. This suggests that E2 administration, to re-establish and maintain circulating physiological concentrations of E2, selectively up-regulates AMPK activity in skeletal muscle of OVX mice. This study also demonstrated that within minutes E2 rapidly activated AMPK in myocytes, suggesting E2 can directly activate AMPK in a non-genomic manner. Importantly, this rapid increase in AMPK phosphorylation was inhibited by a selective antagonist on ERs, thus demonstrating E2 can stimulate rapid and robust AMPK phosphorylation through non-genomic activation of membrane-bound ERs. Conversely, one study demonstrated that despite higher lipid oxidation and similar basal AMPK expression, protein content, and activity, women had lower AMPK activation than men (~200% increase)[167]. However, this study was conducted during the mid-follicular phase when E2 concentrations are low. These findings suggest the mediation of E2 on the beneficial metabolic effects of AMPK may be favourable for lipid metabolism during exercise. In periods of low E2 such as the FP, the slower classical, genomic actions of E2 may allow benefits of an upregulated lipid metabolic pathway and explain consistently observed sex differences in exercise studies which are conducted in the FP[168]. However, in periods of increased E2 concentrations such as the LP, additional non-genomic actions of E2 can maximize the benefits of lipid metabolism[153].

As aforementioned, a recent study in our laboratory demonstrated that in response to repetitive bouts of exercise during proestrus T1DM female rats demonstrated a greater

BG recovery post-exercise than T1DM males[155]. Both T1DM males and females rats repeatedly demonstrated drops in BG throughout the 4-day protocol compared to non-T1DM rats. T1DM rats also demonstrated depleted liver glycogen content; however, T1DM females were able to stabilize BG more effectively post-exercise than T1DM male rats. Therefore, it appears females with T1DM may have a protective mechanism against exercise-induced hypoglycemia that males do not. The mechanism which underlies this proposed acute E2-mediated protective effect in T1DM is unknown, however it is plausible non-genomic activation of AMPK via E2 results in an associated enhancement in lipid oxidation.

1.7 Rationale

Antecedent periods of hypoglycemic stress induced by exercise results in less effective and/or blunted counterregulatory responses to subsequent glycemic challenges[67, 70]. This blunted effect of counterregulatory responses to subsequent hypoglycemia appears to be sexually dimorphic whereby women are better able to preserve the counterregulatory defense than men[65, 69, 70, 72]. Our laboratory recently demonstrated that T1DM females appear to be more protected against hypoglycemia development during repetitive exercise[155]. This may be due to fuel selection differences between the sexes whereby women have an increased reliance on lipid metabolism during exercise thus allowing for quicker normalization of BG concentrations by conserving muscle glycogen and plasma glucose. It appears E2 plays a major role in these differences with consistent evidence demonstrating E2 enhances lipid availability and oxidation[73, 168]. Several studies have demonstrated increased lipid metabolism and reduced glycogen usage during exercise throughout the menstrual cycle, in particular during the LP when E2

concentrations are high[107, 108]. Altogether, in response to repetitive bouts of exercise-induced hypoglycemia, females may demonstrate a fuel selection shift towards lipid oxidation while conserving carbohydrate energy stores resulting in more efficient BG recovery. This proposed fuel selection-associated protective effect may be more pronounced during exercise in the LP.

The underlying mechanisms which explain why hypoglycemia risk may be lower in T1DM females in response to repetitive exercise during proestrus remain to be determined. The mechanistics of E2's metabolic action on lipid and glucose metabolism are not clear but there is evidence to suggest that nongenomic activation of AMPK may mediate its actions[153]. Activation of AMPK increases lipid oxidation which in turn may alleviate glycemic challenge by reducing glucose provision demand and conserving muscle glycogen stores for quicker BG recovery. An E2-mediated shift towards lipid oxidation also suggests glucose producing metabolic pathways would decrease, such as GNG which is regulated by the transcriptional modulation of key rate-limiting enzymes PEPCK and G6Pase[134, 137]. E2 has shown to decrease the expression of PEPCK and G6Pase thereby decreasing GNG. A decrease in GNG may further promote a fuel selection shift towards lipids; however, it is unclear how this may influence T1DM as GNG is the predominant for glucose provision during exercise.

Understanding sex-related differences present in fuel metabolism during different metabolic states and conditions (i.e., menstrual cycle phase, T1DM) could contribute to the development of sex-specific preventive and therapeutic strategies including exercise programs. For individuals with T1DM, recommendations modified for each sex has the potential to assist in achieving and sustaining euglycemia during and following exercise.

1.8 Purpose and Hypotheses

The purpose of this study was to investigate the underlying mechanisms which mitigate the hypoglycemic risk in response to repetitive bouts of aerobic exercise during proestrus in streptozotocin-induced T1DM female rodents compared to males. We examined whether repetitive bouts of exercise-induced hypoglycemia would result in sex-related differences in fuel selection and whether exercise in proestrus maximized the benefits of E2-mediated action on glucose and lipid metabolism. In particular, we examined whether a fuel usage shift towards lipids was present and if muscle glycogen stores were conserved in T1DM females. We also examined AMPK activity and whether there was an enhanced E2-induced activation of AMPK in exercising females. Moreover, we examined whether AMPK activity corresponded to alterations in lipid metabolism through β-oxidation activity measures. Lastly, we examined if key GNG enzymes expression would change in response to an E2-mediated shift in lipid oxidation.

We hypothesized that females with T1DM would have a greater preference towards lipid oxidation during exercise in proestrus than males. Secondly, we hypothesized that AMPK activity would be greater in females as a result of E2-induced activation and would correspond to higher lipid oxidation. Lastly, we hypothesized higher lipid oxidation would correspond to decreased GNG (i.e., PEPCK and G6Pase expression) and this would elicit a conservation of carbohydrate energy stores (i.e., muscle glycogen) in females.

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Chapter 2

2.1 Introduction

Insulin producing β-cells of the pancreas are destructed in an autoimmune reaction in Type I diabetes mellitus (T1DM)[1, 2]. This metabolic disorder results in insufficient endogenous insulin leading to elevated concentrations of blood glucose (hyperglycemia)[2]. In the absence of insulin therapy, T1DM can be fatal and is associated life-threatening complications such as nephropathy, retinopathy, and cardiovascular disease[3]. With the advancement of insulin therapy and increasing regimens available, in

the 1990s the Diabetes Control and Complications Trial compared intensive insulin therapy to conventional therapy[4]. They demonstrated that intensive insulin therapy reduces the associated complications of T1DM more effectively than conventional therapy. However, intensive insulin therapy was also associated with a three-fold increase in hypoglycemia onset in patients[5]. The fear of exercise-induced hypoglycemia is cited as one of the most prominent barriers for participating in regular physical activity in T1DM[6]. Exercise has demonstrated to provide many benefits for the management of T1DM including reduced risk for nephropathy and retinopathy, improved cardiovascular fitness and overall well-being[7]. Therefore, developing safe exercise recommendations for T1DM remains a priority.

At rest and during exercise, tight regulatory mechanisms work to maintain stable BG concentrations to prevent hypoglycemia onset. In individuals with and without T1DM, antecedent bouts of hypoglycemia have been demonstrated to blunt these regulatory responses to subsequent bouts of glycemic challenges[8]. In other words, repetitive bouts of exercise-induced hypoglycemia increase the risk of subsequent hypoglycemia through reduced sensitivity to counterregulatory responses (i.e., neuroendocrine, hormonal). Interestingly, the blunted effects of counterregulatory responses to subsequent exercise of moderate intensity are pronounced two-fold in males[9]. The impact of sex-related differences in counterregulatory responses to successive bouts of exercise on hypoglycemic risk is not well understood in T1DM. A recent study in our lab demonstrated in response to repetitive bouts of aerobic exercise during proestrus T1DM females had a greater post-exercise BG recovery compared to T1DM males despite significant drops in BG throughout the exercise sessions (Appendix

A)[10]. This finding suggests that while there was an equal drop in BG between T1DM females and males, females were able to counteract this drop more successfully than males and may be less susceptible to the blunted counterregulatory responses to repeated exercise-induced hypoglycemia. T1DM females may have different mechanisms to normalize BG concentrations resulting in less risk for hypoglycemia development and quicker BG recovery following exercise.

The role of E2 in fuel metabolism has been well documented and higher E2 concentrations are associated with a shift towards lipid oxidation during exercise[11–13]. In eumenorrheic women, circulating E2 concentrations are highest during the luteal phase (LP), and it has been shown that exercise during this phase results in higher lipid oxidation than during the follicular phase (FP)[14]. Additionally, total and muscle glycogen stores are more preserved after exercise during the LP compared to males as well as females exercising during FP[14]. However, the mechanisms which mediate the action of E2 on fuel selection are not well understood.

A major metabolic enzyme, adenosine monophosphate-activated protein kinase (AMPK), has shown to be non-genomically activated by E2[15]. The activation of AMPK is also induced by exercise resulting in the activation of catabolic processes such as lipid oxidation and inhibition of anabolic processes such as glycolysis[16]. Although recently debated, AMPK has shown to increase lipid availability and oxidation during exercise facilitating a shift towards fats for fuel[16–18]. A shift towards lipid oxidation would suggest a reduced need for glucose provision thus relieving glycemic challenge. For individuals with T1DM, gluconeogenesis (GNG) is the predominant pathway for glucose provision during exercise and it is regulated by the transcriptional modulation of key rate-limiting

enzymes, PEPCK and G6Pase[19]. Reduced expression of these enzymes would suggest GNG is decreased. Interestingly, E2 has also shown to decrease GNG through reduced expression of PEPCK and G6Pase[20]. Although this may indicate a shift towards lipid utilization, it is unclear how E2-mediated actions on GNG may influence exercise-associated fuel metabolism in T1DM females. Therefore, whether periods of increased E2 concentrations provide maximized benefits in lipid metabolism and conserve muscle glycogen stores with a concomitant increase in AMPK during exercise in T1DM is not known. Additionally, it is not understood how exercise during proestrus (high E2) influences the GNG pathway in T1DM. It is plausible exercise during proestrus may protect T1DM females against exercise-induced hypoglycemia due to an E2-mediated shift towards lipid oxidation thereby conserving carbohydrate energy stores (i.e., muscle glycogen) for a quicker BG recovery.

The purpose of this study was to examine the sex-related differences in fuel metabolism to repetitive bouts of aerobic exercise during proestrus in streptozotocin-induced T1DM rats. In particular, we examined whether exercise in proestrus maximized the beneficial metabolic actions of E2 on lipid metabolism. Moreover, to examine E2-mediated action on lipid metabolism AMPK and β-oxidation activity were assessed, as well as to whether a shift towards lipid oxidation would lead to a change in the expression of key enzymes in GNG. We hypothesized a sex-related difference in fuel metabolism would be present such that T1DM females would demonstrate a greater preference towards lipid oxidation during exercise in proestrus than males. In addition, we hypothesized that E2-induced activation of AMPK would correspond to greater lipid

oxidation in females in conjunction with decreased GNG and conserved carbohydrate energy stores (i.e., muscle glycogen).

2.2 Methods and Materials

2.2.1. Experimental Design

This study is a secondary analysis of data from a previous study from our laboratory. The study was originally designed to assess the sexual dimorphism in the blood glucose response to recurrent bouts of prolonged aerobic exercise in T1DM and control rats. Twenty Sprague Dawley rats (10 male, 10 female) were randomly assigned into one of four groups: control (non-T1DM) exercise male (CXM, n = 5), control exercise female (CXF, n = 5), T1DM exercise male (DXM, n = 5), and T1DM exercise female (DXF, n = 5). Diabetes was induced using daily low-dose streptozotocin injections over a one-week period. Insulin pellets were surgically implanted as needed to maintain a blood glucose range of 9-15 mmol/L a target BG range used by exercising individuals with T1DM[10]. The exercise protocol consisted of four consecutive days of treadmill running (1hour, ~75% VO_2 max. or 27m/min per day). Day 4 of exercise was staged during the proestrus stage of the estrous cycle and rats were sacrificed 5 minutes post-exercise. Liver and skeletal muscle tissues were harvested and stored at -80 °C.

2.2.2. Western Blotting

Liver (right lobe) and muscle (red portion of the vastus lateralis) samples were removed from storage at -80°C and kept on ice. Muscle and liver tissue were weighed (20 mg) and submerged in lysis buffer (15 mM Tris pH=7.0, 600 mM NaCl and 0.1mM EDTA) for a 1:10 weight-volume (w/v) ratio of tissue to buffer. The buffer-liver mixture was kept on ice while being subjected to three, 1-3 second pulses by a basic mechanical

homogenizer (IKA Laboratories). Homogenized samples were transferred into 1.5 ml Eppendorf tubes and put on a shaker at 4°C for 2 hours. Afterwards, samples were centrifuged at 4°C at 12 000 rpm for 20 minutes, followed by extraction of supernatant (Appendix D). A Bradford protein assay (Bio-Rad) was used to determine protein concentration (Appendix G). Protein samples were prepared with an equal volume of 2x Laemmli SDS-PAGE (4% SDS, 20% Glycerol, 10% β-mercaptoethanol, 0.015% bromophenol blue, 0.125M Tris, pH 6.8) and submerged in a 90°C water bath for 5 minutes. The protein concentration of samples loaded onto the gels was ensured to be within linear range of the assays when using the specified antibodies. Approximately 15-25 ug of protein was loaded into 12% polyacrylamide gels and ran at 140V for 2 hours. Gels were then transferred on nitrocellulose membranes (Bio-Rad) at 4°C at 70V for 1.5 hours. Following transfer, membranes were washed in TBS-T (Tris Buffer Saline, 0.1% Tween-20) for 5 minutes, 3 times. Membranes were then blocked with a 5% w/v solution of TBS-T and skim milk powder for 1 hour before being washed in TBS-T 3 times, 5 minutes each time. Once washed, membranes were incubated for 2 hours at room temperature with primary antibodies detecting: anti-AMPKα2 (Thr^{172} phosphorylation) (#2531; Cell Signaling Technology, Danvers, MA, USA), anti-AMPKα (#2532; Cell Signaling Technology, Danvers, MA, USA), anti-B-actin (ab8227; Abcam, Cambridge, UK), anti-PEPCK (ab70358; Abcam, Cambridge, UK), and anti-G-6-pase (ab93857; Abcam, Cambridge, UK) per manufacturer's instructions. The following day, primary antibody was removed, and membranes were washed for 10 minutes in TBS-T, 3 times before being incubated with a 5% w/v solution of skim milk powder, TBS-T and secondary antibody (#170-6515 Goat pAB anti-rabbit IgG HRP conjugate; BioRad,

Hercules, CA, USA) at a 1:20000 dilution for 2 hours at room temperature. After incubation, membranes were washed in TBS-T for 10 minutes, 3 times. Washed membranes were then prepared in Bio-Rad chemiluminescence substrate and images were captured using the BioRad Chemidoc MP System. Western blot images were quantified using image processing program Image J and statistical analyses were preformed and interpreted using GraphPad Prism.

2.2.3. Muscle Glycogen Content

2.2.3.1 Muscle Homogenization

Muscle (red portion of the vastus lateralis) samples were removed from storage at -80°C and kept on ice. Approximately 20 mg of tissue was excised from the sample and placed into a 1.5 mL Eppendorf tube (Appendix F). Samples were submerged in 30% potassium hydroxide solution saturated with sodium sulfate and placed in a 90°C water bath for 30 minutes. Samples were precipitated in 95% ethanol for 30 minutes in an ice bath, followed by centrifugation at 3000 rpm for 30 minutes. The supernatant was discarded, and the glycogen pellet immediately dissolved and vortexed in ddH_2O.

2.2.3.2. Glycogen Quantification

Homogenized muscle samples were added to a 96 well uncoated plate. A colour reaction was developed by rapid addition of 5% phenol and 98% sulfuric acid to the sample. Samples were placed in a water bath (37°C) for approximately 20 minutes. Samples were analyzed in triplicate and read at 495 nm on a microplate reader and compared to known glycogen standards (Appendix F).

2.2.4. Muscle Lysate Assessment of β-oxidation (SCHAD Assay)

2.2.4.1. Muscle Homogenization

Muscle (red portion of the vastus lateralis) samples were removed from storage at -80°C and kept on ice. Approximately 30 mg of tissue was excised from the sample and placed into a 1.5 mL Eppendorf tube (Appendix E). Samples were submerged in buffer (5 mM potassium dihydrogen orthophosphate (K_2HPO_4), 1 mM EDTA, 0.1 mM DTT; pH of 7.4) for a 1:10 weight-volume (w/v) ratio of tissue to buffer. The buffer-muscle tissue mixture was kept on ice while being subjected to three, 1-3 second pulses by a basic mechanical homogenizer (IKA Laboratories).

2.2.4.2. β-oxidation activity

Homogenized muscle samples were added to a quartz cuvette with assay buffer (1M Tris-HCl, pH 7.0, 0.5 M EDTA, pH 8.0, 10% Triton X-100) and 5mM NADH. The mixture was incubated for 4 minutes at 30°C allowing for mitochondrial permeability. Following an additional 1 minute after incubation the reaction was initiated by adding 5mM acetoacetyl CoA. The sample cuvette was vortexed, and the reaction was read for 2 minutes in 30 second intervals on a NanoDrop2000 C Spectrophotometer (Waltham, MA, USA) at 340 nm.

2.2.5. Data analysis

Western blot quantification, muscle glycogen content, and β-oxidation activity were analyzed using a two-way analysis of variance (ANOVA), with sex being the between variable and condition (control and diabetes) being the within variable using GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA). When significance

was obtained a post-hoc analysis was performed using Tukey's multiple comparisons test. The level of significance was set at $P = 0.05$. Values presented in tables and text are standard error of the mean (SEM).

2.3 Results

2.3.1. β-oxidation activity

β-oxidation, short-chain β-hydroxyacyl-CoA dehydrogenase (SCHAD) assay was measured in lysates of rat skeletal muscle (red portion of vastus lateralis) to assess lipid oxidation activity. There was a main effect of sex ($P < 0.05$, $P = 0.0326$). There was no interaction between sex and diabetes ($P > 0.05$) (*Fig 1).* There was no effect of diabetes ($P > 0.05$). There was no difference in β-oxidation activity between CXF and CXM ($P > 0.05$) or between DXF and DXM ($P > 0.05$). Overall, females demonstrated greater β-oxidation activity than males.

2.3.2. AMPK expression and activity

Western blot analysis of AMPKα was assessed in lysates of rat skeletal muscle (red portion of vastus lateralis) and demonstrated a main effect of sex ($P < 0.05$; $P = 0.0242$) and diabetes ($P < 0.05$; $P = 0.0244$). There was no interaction between sex and diabetes ($P > 0.05$). There was no difference in AMPKα between CXF and CXM ($P > 0.05$) or between DXF and DXM ($P > 0.05$). AMPKα was significantly lower in DXF compared to CXM ($P < 0.05$; $P = 0.0145$). Overall, males demonstrated greater AMPKα expression than females.

Western blot analysis of phosphorylated AMPKα 2 (p-AMPK), a hallmark of AMPK activation in muscle, was assessed in lysates of rat skeletal muscle (red portion of

vastus lateralis) and demonstrated no effect of sex or diabetes ($P > 0.05$). There was no interaction between sex and diabetes ($P > 0.05$). The ratio of p-AMPKα2 to AMPKα demonstrated no effect of sex or diabetes ($P > 0.05$) suggesting that the activity of AMPKα2 was not different between groups.

2.3.3. Muscle glycogen content

Muscle glycogen content was assessed in lysates of rat skeletal muscle (red portion of vastus lateralis) and demonstrated no effect of sex or diabetes ($P > 0.05$). There was no interaction between sex and diabetes ($P > 0.05$). There was no difference in muscle glycogen between CXF and CXM ($P > 0.05$) or between DXF and DXM ($P > 0.05$).

2.3.4. Gluconeogenic enzymes

Western blot analysis of G6Pase and PEPCK, rate-limiting enzymes in the gluconeogenesis pathway, were assessed in lysates of rat liver tissue. G6Pase demonstrated a main effect of sex ($P < 0.05$; $P = 0.0080$). There was no effect of diabetes ($P > 0.05$). There was no interaction between sex and diabetes ($P > 0.05$). G6Pase was lower in both CXM and DXM compared to CXF ($P < 0.05$; $P = 0.0173$; $P = 0.0262$). There was no effect of sex or diabetes in PEPCK content ($P > 0.05$). There was no interaction between sex and diabetes in PEPCK content ($P > 0.05$). Overall, females demonstrated greater G6Pase expression than males.

2.3.5 Blood glucose concentrations

Refer to Appendix A. Previous findings from our laboratory conclude on day 4 of exercise DXF demonstrated a quicker post-exercise BG recovery compared to DXM despite similar drops in BG throughout the exercise session.

2.3.6. Estrogen concentrations

Refer to Appendix B. Previous findings from our laboratory verified circulating E2 serum concentrations by comparing day 1 to day 4 concentrations to confirm the proestrus phase on day 4.

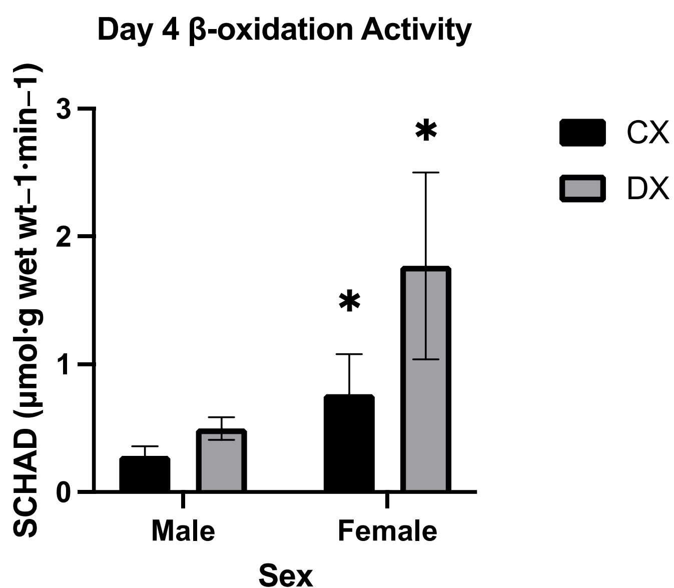
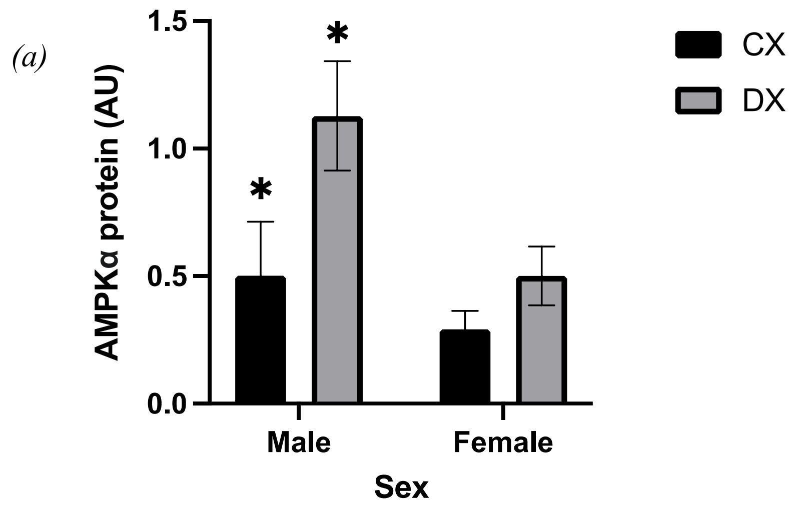
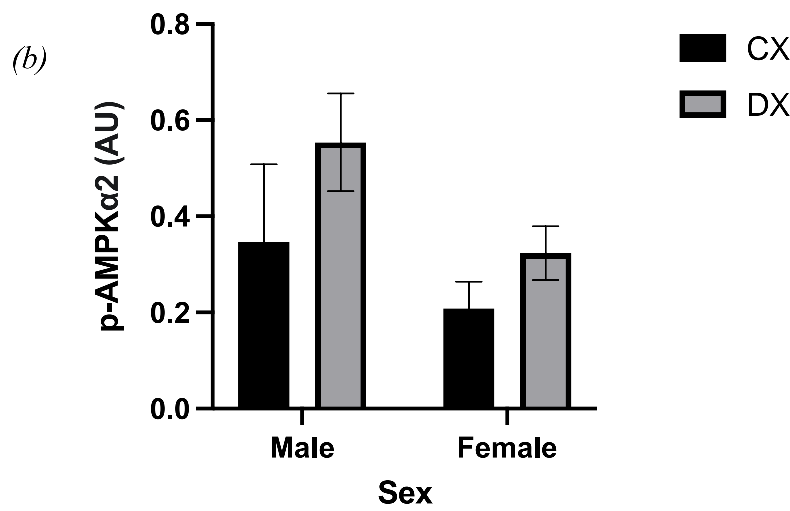


Figure 1. *β-oxidation, SCHAD activity assessment of skeletal muscle in male and female T1DM and control rats. Mean muscle SCHAD activity (µmol·g wet wt^{-1}·min^{-1}) from day 4. All data are presented as mean ± SEM. There was a main effect of sex. DXF and CXF demonstrated greater β-oxidation activity than DXM and CXM. *Denotes significance.***

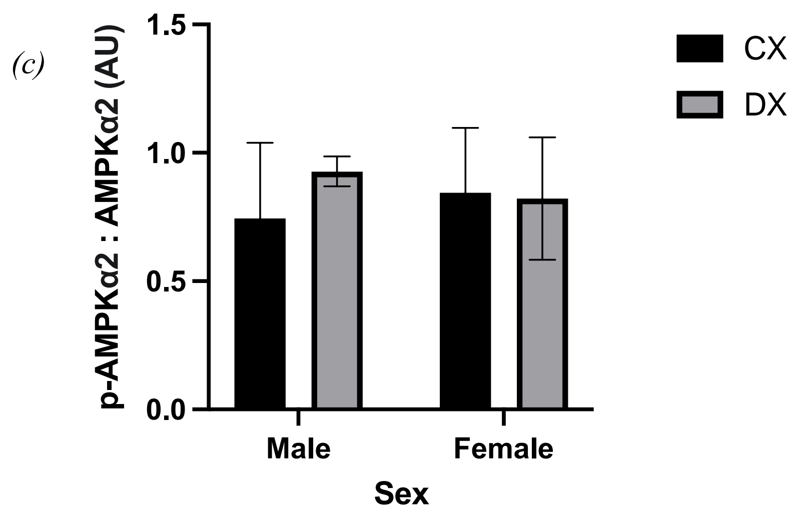
(a)



(b)



(c)



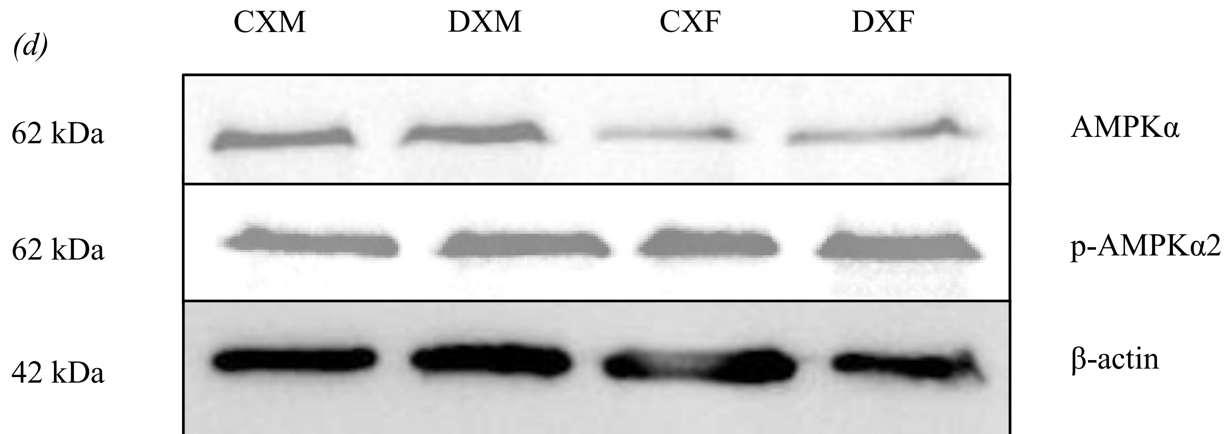


Figure 2. *Western blot quantification and representation blots of AMPKα and p- AMPKα2 from day 4 of exercise protocol for control and diabetic exercise males and females. (a) AMPKα (b) p- AMPKα2 (c) Phosphorylated ratio and (d) Representative blots relative to β-actin control. All data are presented as mean ± SEM. * denotes significance. There was a main effect of sex (P = 0.0242) and diabetes (P = 0.0244) for AMPKα. There was a difference between DXF and CXM in AMPKa (P = 0.0145). There was no difference in AMPKa between CXF and CXM (P > 0.05) or between DXF and DXM (P > 0.05). There were no significant differences in p- AMPKα2.*

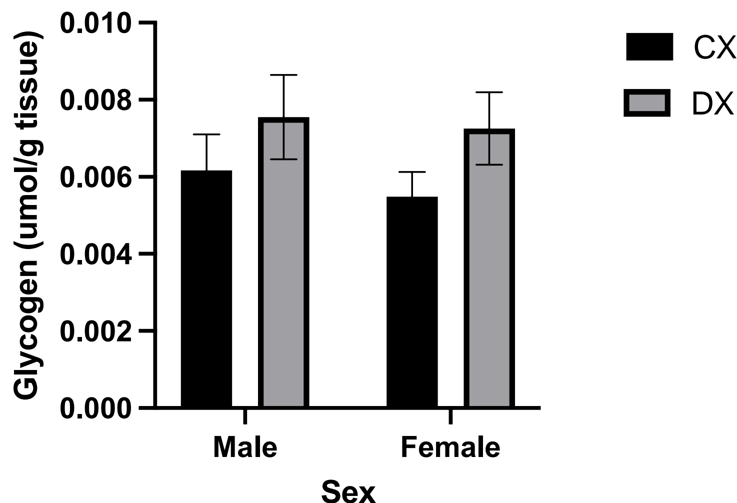
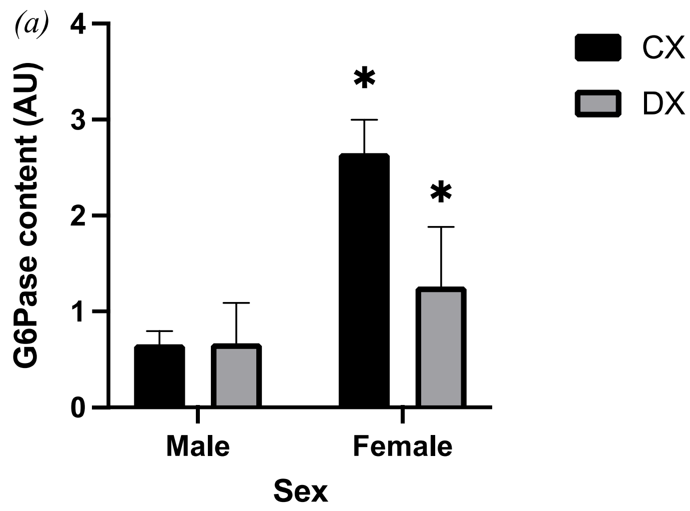
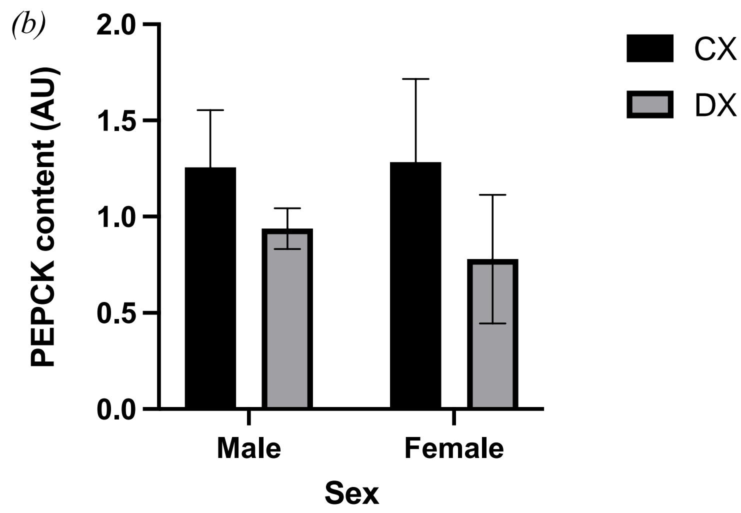


Figure 3. *Muscle glycogen content measured in umol/g from day 4 of exercise for control and diabetic exercise males and females. All data are presented as mean ± SEM. There was no significant difference between CXF and CXM or between DXF and DXM (P > 0.05).*


(a)
G6Pase content (AU)
4
3
2
1
0
*
*
Male
Female
Sex
CX
DX


(b)
PEPCK content (AU)
2.0
1.5
1.0
0.5
0.0
Male
Female
Sex
CX
DX

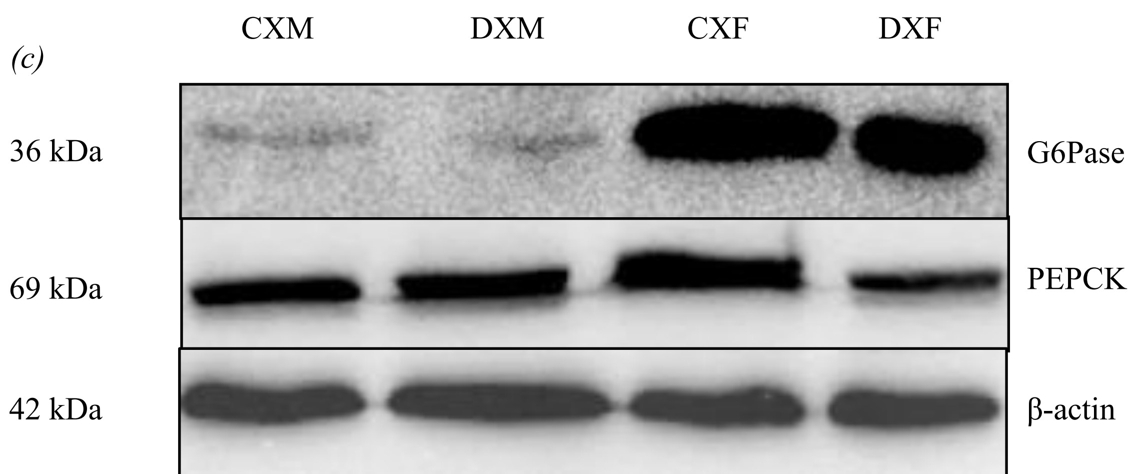


Figure 4. *Western blot quantification and representative blots for gluconeogenic enzymes G6Pase and PEPCK. (a) G6Pase protein content quantification (b) PEPCK protein content quantification and (c) Representative blots relative to β -actin control. All data are presented as mean ± SEM. * denotes significance ($P < 0.05$). There was a main sex effect in G6Pase ($P = 0.0080$). DXM ($P = 0.0173$) and CXM ($P = 0.0262$) were significantly lower than CXF. There were no significant differences in PEPCK.*

2.4 Discussion

Extensive reviews and commentaries have been published to recommend safe exercise practices and strategies for T1DM management[21–25]. Yet, individuals with T1DM are still disproportionately inactive and cite the fear of hypoglycemia as a main barrier to regular physical activity[6, 23]. Research efforts examining the role for exercise in the management of T1DM has focused predominantly on male subjects. Most recent findings in our laboratory demonstrated that T1DM females had a better post-exercise BG recovery compared to T1DM males in response to repetitive bouts of aerobic exercise in proestrus (Appendix A)[10]. While T1DM males and females presented significant drops in BG throughout the course of exercise, it appears females were able to stabilize BG concentrations post-exercise more efficiently than T1DM males. This finding suggests

that T1DM females may be less susceptible to the blunted counterregulatory responses to subsequent exercise-induced hypoglycemia. Moreover, this finding suggests that T1DM females may be less susceptible to hypoglycemia development during exercise in proestrus due to the metabolic actions mediated by E2.

It has been postulated women are better at maintaining BG homeostasis during exercise due to sex-related differences in the fuel selection[9, 11]. The role of E2 is increasingly recognized in many physiological systems including the glucose counterregulatory responses to exercise. E2 influences fuel metabolism during exercise and is associated with a shift in fuel usage towards lipid oxidation thereby preserving carbohydrate energy sources for quicker BG recovery[11–13, 26]. Therefore, the purpose of this study was to investigate the underlying mechanisms which explain why T1DM females recover more efficiently during repetitive acute aerobic exercise in proestrus, particularly the sex-related differences in fuel metabolism.

The results of the current study demonstrate that there is a sex-related difference in lipid oxidation during exercise in females. Both T1DM and non-T1DM females demonstrated significantly higher β-oxidation activity in skeletal muscle immediately after exercise compared to T1DM and non-T1DM males (*Figure 1)*. This would suggest a shift in fuel usage towards lipid oxidation in both T1DM and non-T1DM females during repeated acute moderate-intensity aerobic exercise. We hypothesized that greater lipid oxidation in females would correspond to increased AMPK activity as a result of E2-induced activation; however, we found no differences in AMPK activity (AMPKα2 phosphorylation) between the sexes (*Figure 2b* and *2d)*. AMPKα expression demonstrated a diabetes effect and was higher in males (*Figure 2a)*. These findings agree

with previous work demonstrating higher rates of lipid oxidation in females during exercise in the FP is not associated with a concomitant increase in AMPKα2 activity compared to males[27]. This may suggest that females have greater control over energy balance and increased lipid oxidation is not exclusively mediated by AMPKα2 during exercise, which has also been supported by recent evidence[18]. Additionally, our findings suggest AMPKα2 expression and activation are not necessarily correlated with higher E2 concentrations thus the mediated actions of E2 on lipid metabolism during exercise may be through other signaling pathways. A study demonstrated an increase in AMPK activity in exercising men compared to women similarly to the findings presented here, an increase in AMPKα expression in T1DM and non-T1DM males (*Figure 2a)*[27]. However, the increase in AMPK activity was associated with an increase in lipid oxidation which was not evident in the males in the current study. An increase in AMPKα expression in T1DM and non-T1DM males may suggest males require increased concentration of AMPKα expression to elicit the same activation as females.

We hypothesized that T1DM and non-T1DM females would demonstrate a greater preference towards lipid oxidation during exercise in proestrus and that this E2-mediated fuel shift would in turn conserve muscle glycogen stores. We found no differences between the sexes in muscle glycogen content (*Figure 3)*. In humans without diabetes, the LP is associated with less muscle glycogen mobilization during aerobic exercise suggesting less plasma glucose uptake or carbohydrate intake may be required for post-exercise glycogen replenishment[14]. As a result of no baseline muscle glycogen content our findings cannot verify if there was a difference in muscle glycogen utilization, nor can we confirm if there were phase specific differences in muscle

glycogen use. The studies which have concluded a menstrual phase effect on sparing muscle glycogen content were conducted in isolated bouts of exercise, therefore it can be postulated there was no muscle glycogen differences between the sexes due to the exhaustive nature of the prescribed exercise protocol. Successive days of exercise have demonstrated to produce a marked reduction and utilization of muscle glycogen content. In particular, a study investigated a 3-day running protocol on muscle glycogen content and utilization[28]. On day 1, pre-exercise muscle glycogen content was approximately 2.1 g/100g and 1.4g/100g post-exercise. However, on the third consecutive day of running, muscle glycogen content pre-exercise was reduced to 1.3 g/100g and approximately 0.7 g/100g post-exercise. Muscle glycogen content of our rats on day 4 post-exercise ranged from approximately 0.25 g/100 g to 0.64 g/100g which is substantially lower than the muscle glycogen content post-exercise on day 3 in the aforementioned study. These values suggest that on day 4 the exercised rats in the present study were likely depleted of muscle glycogen. Indeed, post-exercise muscle glycogen stores can take up to >24 hours for complete restoration to near pre-exercise concentrations[29]. Therefore, the successive exercise protocol likely did not allow for full resynthesis of stores and a sparing effect regardless of the mediated action of E2 was difficult to achieve.

Our lab has previously demonstrated that liver glycogen content is significantly depleted in T1DM males and females coinciding with significant drops in BG throughout exercise compared to the control groups (Appendix C). We hypothesized a decrease in hepatic GNG in females as a result of higher E2 concentrations. Interestingly, hepatic gluconeogenic rate limiting enzyme G6Pase was significantly higher in T1DM and non-T1DM females, but no difference between groups was seen in PEPCK content (*Figure*

4). This may suggest that hepatic GNG was in part increased in females as a result of increased precursor availability of glycerol from the breakdown of stored triacylglycerols. Enhanced E2-induced lipid oxidation in females suggests an increase in lipid availability from increased lipolysis leading to higher circulating gluconeogenic precursors free fatty acids and glycerol. The pathway of glycerol into GNG is short and bypasses the tricarboxylic acid cycle and thus G6Pase content is primarily relevant for its conversion into glucose[30, 31]. It can therefore be suggested higher G6Pase expression in females may reflect increased GNG from glycerol. As tissues were harvested during proestrus (Appendix B), it appears a E2-mediated shift towards lipid oxidation in T1DM and non-T1DM females did not correspond to decreased GNG, rather higher precursor availability may have increased GNG through increased G6Pase expression.

Our findings in PEPCK content may also suggest GNG from other precursors such as lactate and amino acids is equal between groups during exercise, however we cannot verify this with our data set. PEPCK content is similar between all groups suggesting the predominant precursor for GNG during exercise in T1DM males may be different than T1DM females. This may be supported by a notable sex-difference in rodent models for gene expression of PEPCK and G6Pase whereby males demonstrate higher basal content of both enzymes[32]. The rate of hepatic glucose output during exercise is predominantly balanced through GNG in T1DM and our findings suggest this pathway may be more upregulated in T1DM females due to increased lipid oxidation[33]. This may be one mechanism which can explain why T1DM females demonstrate a greater resistance to the blunting effects of the counterregulatory responses to repeated exercise-induced hypoglycemia during proestrus.

2.5 Conclusion

Both T1DM and non-T1DM females demonstrated higher lipid oxidation compared to male counterparts. This shift corresponded to elevated concentrations of E2 as exercise was performed during proestrus. We hypothesized that greater lipid oxidation would correspond to conserved muscle glycogen content, however we found no difference between the sexes. This is likely due to depleted glycogen stores in all groups following successive days of high intensity aerobic exercise. Our findings also demonstrate that the shift towards lipids as fuel during exercise did not correspond to higher AMPK activity. Therefore, greater lipid oxidation in females during repetitive bouts of exercise in proestrus cannot be exclusively explained through an AMPK mechanism. The influence of elevated E2 during proestrus did not negatively impact GNG, rather the E2-mediated shift towards lipid oxidation increased the enzyme G6Pase in T1DM and non-T1DM females. An increase in lipid availability likely increased the availability of glycerol as a GNG precursor. As a result, increased GNG in T1DM females may have supported glucose provision leading to more stable BG concentrations post-exercise.

In conclusion, a sex-related difference in lipid metabolism was evident and T1DM females demonstrated a shift towards lipid oxidation in response to repetitive aerobic exercise during proestrus. However, this preference towards lipid oxidation was not accompanied by greater AMPK activity or conserved muscle glycogen stores. Therefore, T1DM females may demonstrate greater resistance to the blunting effects of repeated exercise-induced hypoglycemia due to enhanced use of lipids as fuel for exercising muscle and increased use of GNG precursors (i.e., glycerol) during exercise in proestrus.

Future directed efforts to study sexual dimorphism in T1DM and lipid/glucose metabolism would benefit from measurement of muscle glycogen content at baseline and post-exercise (>24h). In addition, concentrations of blood catecholamines and hormones such as GH would be beneficial to measure pre- and post-exercise due to its role in lipid metabolism and on G6Pase expression. Lastly, future studies should prescribe a less intensive exercise protocol to allow for sufficient muscle glycogen resynthesis thus providing opportunity for a potential sparing effect.

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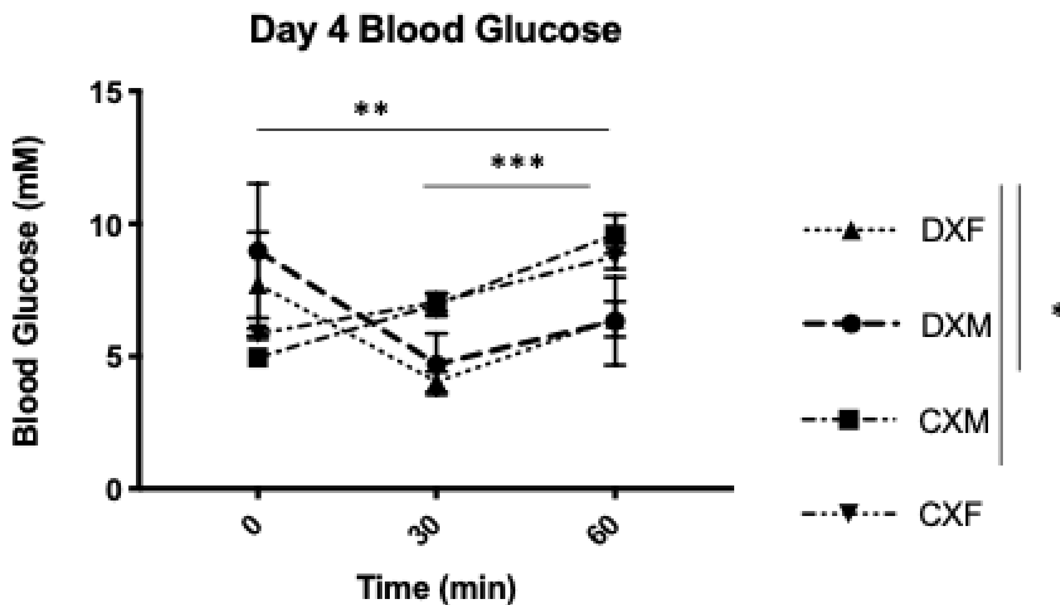
Appendices

Appendix A: Mean blood glucose response from day 4 of exercise protocol.



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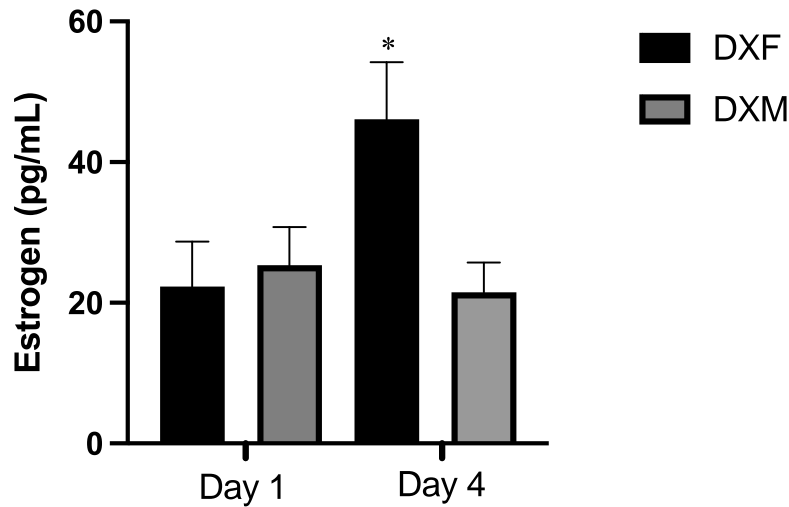
Appendix B: Serum estrogen concentrations from day 1 and day 4 of exercise protocol.



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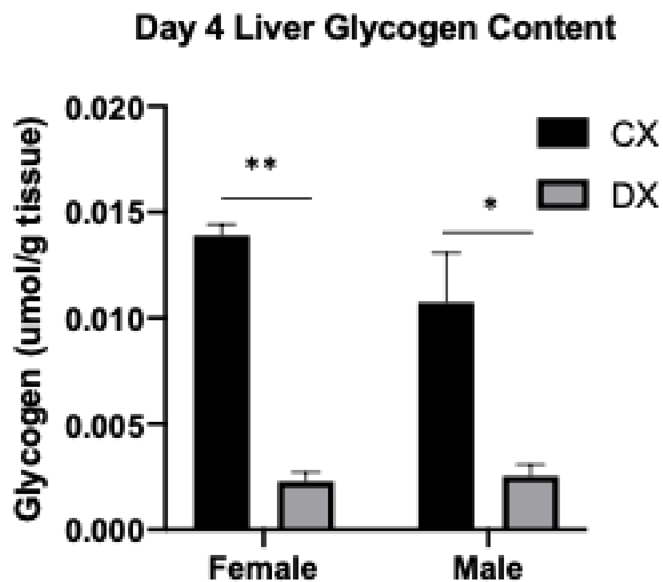
Appendix C: Liver Glycogen Content. Previous findings from our laboratory conclude on day 4 of exercise liver glycogen content was significantly depleted in diabetes groups compared to control groups.



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Appendix D: Western Blotting Protocol. Western blotting method for the quantification of protein expression and phosphorylation of AMPKa, p-AMPKa2, G6Pase, and PEPCK.

Tissues tested: Skeletal muscle and liver

Sample preparation:

1. Place homogenized samples on ice.
2. Load the amount of protein in each well previously determined by a protein loading curve.
3. The volume of homogenate needed is determined from a Bradford protein quantification assay (See Bradford Assay protocol).
4. Label a new set of Eppendorf tubes with the appropriate sample names.
5. Dilute the volume of sample in *sample buffer* (1:1 ratio) and vortex.
6. Boil sample and buffer mixture for up to 5 minutes at 90°C.
7. Prior loading samples into gel allow samples to return to room temperature. It may be necessary to vortex or quickly centrifuge samples before loading.

Preparation of gels:

1. Clean short glass plates with 70% ethanol before use and then prepare gel cassette.
2. Prepare *separating gel* according to chart relative to the number of gels and percent acrylamide to be used (*12% separating gel* recipe was used for all blots in this study).

12 % Gel				
	2 gels	**4 gels**	**6 gels**	**8 gels**
ddH$_2$O	6.663 mL	13.325 mL	19.988 mL	26.65 mL
Acrylamide solution[1]	8 mL	16 mL	24 mL	32 mL
Separating gel buffer[2]	5 mL	10 mL	15 mL	20 mL
SDS solution[3]	200 µL	400 µL	600 µL	800 µL
10% APS solution	125 µL	250 µL	375 µL	500 µL
TEMED	12.5 µL	25 µL	37.5 µL	50 µL
Total Volume	20 mL	40 mL	60 mL	80 mL

3. After 10 minutes of mixing, pour *separating gel* using Pasteur pipette while trying to eliminate bubbles.

4. Immediately overlay the gel with water saturate isobutanol to ensure a continuous charge from *separating* to *stacking gel.*
5. Wait approximately 45-60 minutes for gel to polymerize and rinse off overlay solution with ddH2O and dry clean with filter paper when *stacking gel (4%)* is ready to pour.
6. Prepare and pour *stacking gel (4%)* according to the chart below:

4 % Gel				
	2 gels	**4 gels**	**6 gels**	**8 gels**
ddH$_2$O	5.992 mL	11.983 mL	17.975 mL	23.967 mL
Acrylamide solution[1]	1.333 mL	2.667 mL	4 mL	5.333 mL
Stacking gel buffer[5]	2.5 mL	5 mL	7.5 mL	10 mL
SDS solution[3]	100 µL	200 µL	300 µL	400 µL
10% APS solution	62.5 µL	125 µL	187.5 µL	250 µL
TEMED	12.5 µL	25 µL	37.5 µL	50 µL
Total Volume	10 mL	20 mL	30 mL	40 mL

7. Place the correct sized comb between the glass plates, ensuring no air bubbles are trapped in the wells continue to pour *stacking gel* mixture on the ends of the comb.
8. Prepare 1L of 1x *running buffer* per 2 gels and store in the refrigerator.
9. Once the *stacking gel* has polymerized (30 minutes), gentle remove comb and fill wells with 1x *running buffer*.
10. Load correct amount of sample and ladder using micropipette with loading tip.
11. Once loading is complete place gels in running unit.
12. Fill running unit with cold 1x *running buffer* (chamber inside the cassette and the outside).
13. Run gels at 70V until through the stacking portion of the gel (~30 minutes) and then 125-130V until sample dye has reached the front of the glass.
14. During the running period prepare *transfer buffer* and keep in refrigerator.

Transfer of Gels to Nitrocellulose:

1. Cut filter paper and nitrocellulose to appropriate size (short plate size).
2. Soak filter paper, nitrocellulose, and Brillo pads in cold *transfer buffer* for 20 minutes.
3. Once running period is complete, assemble the transfer apparatus (“sandwich”) as shown below, making sure to remove all air bubbles between gel and nitrocellulose paper (keep sandwich completely submerged in *transfer buffer* at all times).
4. Place “sandwich” into transfer holding tank making sure the black, negative side is facing the black transfer unit. Fill tank with cold *transfer buffer* and add ice pack into the unit to keep transfer period cold throughout.
5. Connect to power supple and run at 70V for 1.5 hours.

Blocking:

1. Prepare 1L 1x *TBS* per 2 blots.
2. After transfer, gently remove gel and place in small container with *5% blocking solution* (optional, rinse gel once with 1x TTBS for 5 minutes before blocking). Incubate up to 2 hours on shake at room temperature.
3. After blocking prepare primary antibody (minimum 20ml of solution).
4. Wash blots 1x in TTBS for 5 minutes.
5. Incubate blots in primary antibody solution overnight at 4°C or for two hours at room temperature.
6. Once finished, the primary antibody solution can be stored in the refrigerator for use within a week or stored in the freezer for long term storage.
7. Wash blots 3x in TTBS for 10 minutes each.
8. Prepare secondary antibody (confirm HRP, not AP) solution (1:5000-25000).
9. Incubate blots for 1 hour on shaker at room temperature.
10. Wash blots 3x in TTBS for 10 minutes each. Keep in 1x TBS for long-term storage.

Western Blotting Solutions:

	Name	Components, concentrations, pH	Example amounts	Storage
1.	Acrylamide solution	Acrylamide – 30% (w/v) Bis-acrylamide – 0.8% (w/v)	Acrylamide – 150 g Bis-acrylamide – 4 g Total V - 500 mL	4°C
2.	Separating gel buffer	Tris – 1.5 M SDS – 0.4 % (w/v) pH – 8.8	Tris – 90.9 g SDS – 2 g Total V- 500 mL	4°C
3.	SDS solution	SDS – 10% (w/v)	SDS – 10 g Total V - 100 mL	Room temperature
4.	2X Laemmli SDS-PAGE sample buffer	Tris - 0.125 M Glycerol – 20% (v/v) SDS – 4% β –mercaptoethanol – 10% (v/v) Bromophenol blue – 0.015% (w/v) pH – 6.8	Tris – 7.57 g Glycerol – 100 mL SDS – 20 g β –mercaptoethanol – 50 mL Bromophenol blue – 0.075 g Total V – 500 mL	Room temperature, fumehood
5.	Stacking gel buffer	Tris – 0.5 M SDS – 0.4% (w/v) pH – 6.8	Tris – 30.3 g SDS – 2 g Total V – 500 mL	4°C
	10X running buffer	Tris – 0.25 M Glycine – 1.92 M SDS – 1% (w/v)	Tris – 60.6 g Glycine – 288 g SDS – 20 g Total V – 2 L	4°C

6.	1X running buffer	10X running buffer – 10% (v/v)	10 X running buffer – 100 mL Total V – 1 L	4°C
7.	1X transfer buffer	10X running buffer – 10% (v/v) Methanol – 20% (v/v)	10X running buffer – 100 mL Methanol – 200 mL (add last) Total V – 1 L	4°C or colder
	10X TBS	Tris – 0.1 M NaCl – 1 M pH – 7.5	Tris – 24.2 g NaCl – 116.9 g Total V – 2 L	Room temperature
8.	1X TTBS	10X TBS – 10% (v/v) Tween-20 – 0.1% (v/v) pH – 7.5	10X TBS – 200 mL Tween-20 – 2 mL Total V – 2 L	Room temperature
9.	Blocking solution	**a.** Non-fat, dry milk protein – 5% (w/v) **b.** Bovine serum albumin – 5% (w/v) 1X TTBS – 95%	**a.** Non-fat, dry milk protein – 1.25 g **b.** Bovine serum albumin – 1.25 g 1X TTBS – top up to 25 mL	Room temperature
10.	Primary antibody solution 1	Non-fat, dry milk protein – 2% (w/v) 1X TTBS – 98% Antibody against protein of interest	Non-fat, dry milk protein – 0.4 g 1X TTBS – top up to 20 mL	4°C (-20°C long term)
11.	Primary antibody solution 2	Bovine serum albumin – 5% (w/v) 1X TTBS – 95% Antibody against protein of interest	Bovine serum albumin – 1 g 1X TTBS – top up to 20 mL	4°C (-20°C long term)
12.	Secondary antibody solution	Non-fat, dry milk protein – 2% (w/v) 1X TTBS – 98% Antibody against primary antibody	Non-fat, dry milk protein – 0.4 g 1X TTBS – top up to 20 mL	Room temperature
13.	1X TBS	10X TBS – 10% (v/v) pH – 7.5	10X TBS – 10 mL Total V – 100 mL	Room temperature

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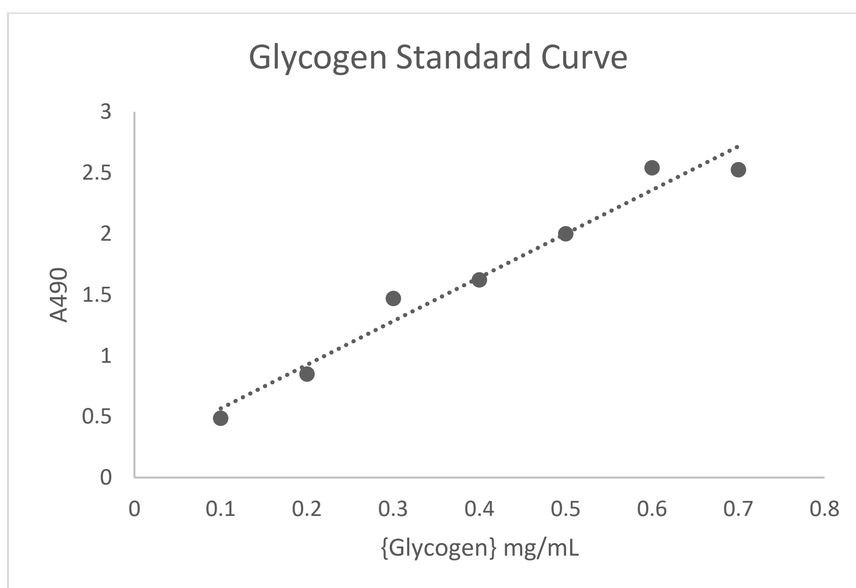
Murray, Michael, "The Effect of Aerobic Exercise Training on Hepatic Glycogen Metabolism in Type 1 Diabetic Rats" (2015). *Electronic Thesis and Dissertation Repository*. 2726. https://ir.lib.uwo.ca/etd/2726

Appendix E: Phenol-Sulphuric Acid Assay. Glycogen content quantification was assessed with the phenol-sulphuric acid assay.

Part A: Solutions

1. **30% KOH (w/v)**
   a. 30 g of KOH pellets
   b. 100 mL of ddH_2O
   c. Exothermic reaction — combine in an Erlenmeyer flask placed in an ice bath.
2. **95% Ethanol (v/v)**
   a. 95 mL of 100% Ethanol
   b. 5 mL of ddH_2O
3. **Glycogen Standards**
   a. 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.7 mg/mL
   b. Prepare large volumes of standards and store in 15 mL Falcon Tubes
   c. Standard curve flattens out after 0.7 mg/mL (see below)[1]
      i. Linear portion spans from 0 to 0.6 or 0.7 mg/mL



4. **100% Sulfuric Acid (stock)**
5. **5% Phenol (v/v)**
   a. 5 mL of 100% phenol (stock)
   b. 95 mL of ddH_2O

Part B: Tissue Homogenization and Glycogen Isolation

1. Cut and weigh approximately 20 mg of tissue, place into a 2.0 mL Eppendorf tube and keep on ice until the next step.
2. For 12 samples, saturated ~10 mL of 30% (w/v) KOH solution with Na_2SO_4.
3. Boil approximately 0.75 L of water and pour into the metal tray on the hot plate. Set the hot plate to MAX.

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[1] With a 5% (w/v) Phenol solution, the standard curve was linear from 0.0 – 1.0 mg/mL glycogen concentrations with an $R^2 = 0.97$.

4. Add 500 µL of the Na_2SO_4-saturated KOH solution to each Eppendorf tube, ensuring that each sample is completely submerged.
5. Place samples in a boiling water bath for 30 minutes. Halfway through, agitate the tubes until no pieces of tissue are visible.
6. Place tubes on ice and precipitate glycogen by adding 1 mL of 95% ethanol for 30 minutes.
7. Centrifuge tubes at 3000 rpm for 30 minutes.
8. Discard the supernatant and *immediately* dissolve the pellet in 1 mL of ddH_2O.

Part C: Glycogen Quantification

1. Turn on the hot water bath and set it to ~70 °C.
2. In a flat-bottom polystyrene 96-well microplate, pipette 50 uL aliquots of ddH_2O, glycogen standards and sample glycogen solutions in triplicate.
3. Add 150 µL of sulfuric acid to each well.
4. Quickly add 30 µL of 5% phenol to each well.
    a. Works best with a repeater pipette. Use a 5 mL CombiTip set to 150 µL for the sulfuric acid, and a 0.5 or 1.0 mL CombiTip set to 30 µL for phenol.
5. Cover plate in ParaFilm and place in a static hot water bath for 10 minutes.
6. Dry the microplate with KimWipes and measure absorbance using a microplate reader at 490 nm (1.0 seconds).
7. Calculate glycogen concentration (units: µmol of glycogen/g of tissue):

$$\frac{\mu\text{mol of glycogen}}{\text{g of tissue}} = \frac{\text{A490}}{\text{k} \times \text{W} \times \text{MM}_{\text{Glycogen}}}$$

Where:
A490 = Adjusted Absorbance at 490 nm (Sample Absorbance – Blank Absorbance)
k = slope of the standard curve (units μg^{-1})
W = mass of tissue sample used
$MM_{Glycogen}$ = molar mass of glycogen (666.5777 g/mol)

References

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Appendix F: β-oxidation, short-chain β-hydroxyacyl-CoA dehydrogenase (SCHAD) Assay. Muscle lysate assessment of β-oxidation activity of control and T1DM subjects in red portion of vastus lateralis tissue.

Part A: Solutions

1. **Lysis buffer:**
    - **a.** Measure: 5 mM potassium dihydrogen orthophosphate (K_2HPO_4), 1 mM EDTA, and 0.1 mM DTT.
    - **b.** Adjust to a pH of 7.4.
2. **SCHAD Buffer (50mM Tris-HCl, 1mM EDTA):**
    - **a.** Make stock 0.5M EDTA (pH 8.0):
        - **i.** Measure 146.12g; add to 400 ml of ddH2O.
        - **ii.** Add very concentrated NaOH dropwise until pH 8.0 (colour reaction: white to translucent).
        - **iii.** Bring to 500 ml ddH2O.
    - **b.** Make 1M Tris-HCl (pH 7.0):
        - **i.** Measure 157.6g; add to 1L ddH20.
        - **ii.** Adjust pH to 7.0.
    - **c.** Make *working* buffer (~250 sample runs):
        - **i.** 100 mL ddH2O
        - **ii.** 10 mL *stock* Tris-HCl buffer (pH 7.0)
        - **iii.** 400ul *stock* 0.5 EDTA buffer (pH 8.0)
        - **iv.** Check solution pH (7.0)
        - **v.** Bring solution to 200 mL with ddH2O.
3. **5mM acetoacyl CoA (stored at -20°C).**
    - **a.** 5 mg vial reconstituted in 1.03 mL ddH2O.
4. **10% Triton X-100.**
    - **a.** 5 mL Triton X-100 diluted in 45 mL ddH2O (stored at room temperature).
5. **5 mM NADH (stored at 4°C).**
    - **a.** Reconstitute 3.54mg of sample in 1 mL ddH2O.
    - **b.** Make fresh *daily* and keep on ice.
6. **Assay buffer (stored at 30°C, made fresh daily):**
    - **a.** 1 mL Triton X-100
    - **b.** 49 mL SCHAD buffer

Part B: Muscle homogenization

1. Place approximately 30 mg of muscle tissue and place into a 1.5 mL Eppendorf tube on ice.
2. Submerge sample in lysis buffer for a 1:10 weight-volume ratio.
3. Keep buffer-muscle mixture on ice while subjecting to three, 1-3 second pulses by a mechanical homogenizer.

Part C: Procedure

1. Turn on spectrophotometer and water bath at least 20 minutes prior start. Assay carried out at 30°C.

2. Prepare *assay buffer* fresh *daily* and keep in water bath at 30°C.
3. "Zero" prior sampling by placing blank cuvettes with only assay buffer.
4. Prepare *quartz cuvette* (340 nm wavelength cannot penetrate disposable plastic cuvettes), pipetting repeatedly slowly:
    a. 800 uL assay buffer
    b. 10 uL NADH
    c. 35 uL sample
5. Mixture should be incubated for *4 minutes* at 30°C to allow for mitochondrial permeability in water bath.
6. Start measurement routine following incubation period. Wait an additional 1 minute following the 4-minute incubation period (a total of 5 minutes pre-assay incubation).
7. Initiate reaction by adding 10 uL of acetoacetyl CoA.
8. Mix sample cuvette using pipette.
9. Read for 2 minutes, every 30 seconds.
10. Calculate:
    a. Determine the specific activity (SA):
        i. SA = [ΔAbs / volume assayed (mL)] / $6.3*10^{-3}$ nmol*min^{-1}
    b. Total amount of protein available in the homogenate
        i. = [protein] mg/mL * mass of wet wt. of tissue * 0.025
    c. Total number of units = SA * total amount of protein
    d. Units/wet weight = total # of units / total mass of tissue homogenized.

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Tarnopolsky, M. A., Rennie, C. D., Robertshaw, H. A., Fedak-Tarnopolsky, S. N., Devries, M. C., & Hamadeh, M. J. (2007). Influence of endurance exercise training and sex on intramyocellular lipid and mitochondrial ultrastructure, substrate use, and mitochondrial enzyme activity. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, *292*(3). https://doi.org/10.1152/ajpregu.00472.2006

Appendix G: Bradford Protein Assay. Analytical procedure used to calculate the concentration of protein in liver and muscle samples.

Procedure:

1. Dilute 1 part dye reagent with 4 parts ddH2O (Bio Rad 500-0006). One microplate requires 25 mL reagent.
2. Filter the diluted solution through Whatman 1 filter paper (store solution at room temperature up to two weeks).
3. Add the indicated amounts of BSA (1 mg/mL) or unknown protein sample, water, and reagent respectively into a 96-well microplate.

Protein (ug)	**Water (uL)**	**BSA (uL)**	**Unknown sample (uL)**	**Reagent (uL)**
0	10	0	-	200
1	9	1	-	200
2	8	2	-	200
3	7	3	-	200
4	6	4	-	200
5	5	5	-	200
6	4	6	-	200
unknown	9.5	-	0.5	200

4. Shake and incubate at room temperature for a couple of minutes.
5. Read absorbance at 595 nm.

References

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1–2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

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